

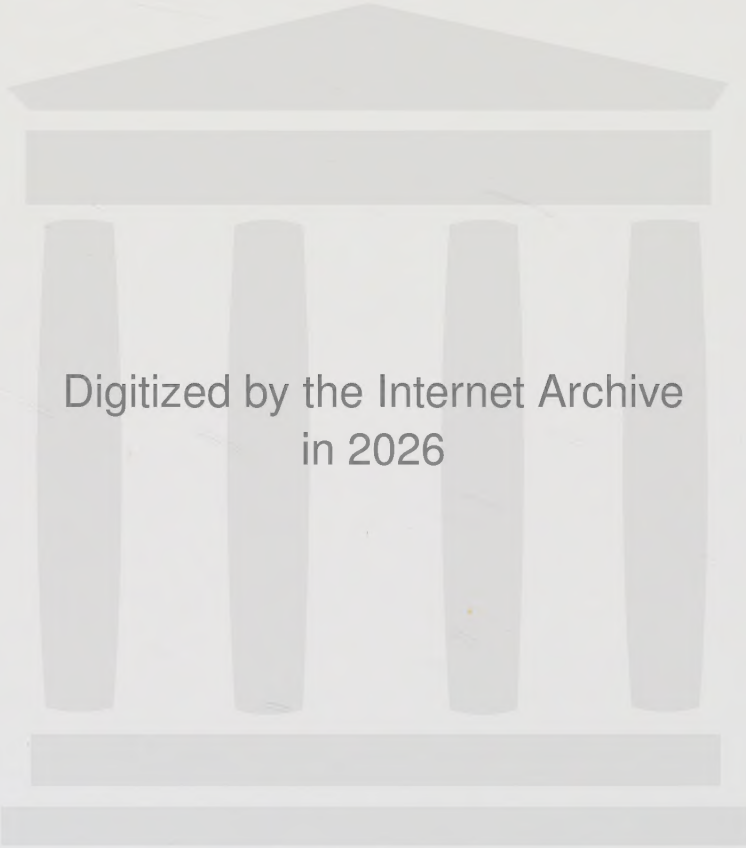
α_1 -microglobulin

Studies in guinea pig and man

Bo Åkerström



Lund 1982



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α_1 -MICROGLOBULIN
STUDIES IN GUINEA PIG AND MAN

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Abstract α_1-microglobulin was purified from urine of guinea pigs with sodium chromate-induced proteinuria. It was characterized as a glycoprotein with a heterogeneous net charge, and strongly associated with a brown-colored chromophore. It was detected in relatively low concentrations in guinea pig plasma, where it also appeared in high molecular weight complexes. Guinea pig α_1-microglobulin and human α_1-microglobulin cross-reacted immunologically. The amounts of α_1-microglobulin in some guinea pig tissues were investigated. The ratio α_1-microglobulin to albumin was elevated in kidneys and liver, as compared to plasma, adrenal glands, brain, heart, lungs, muscle, placenta, spleen, and testes. Isolated guinea pig liver cells released α_1-microglobulin when cultured <i>in vitro</i>. A <i>de novo</i> synthesis of α_1-microglobulin was demonstrated in guinea pig liver explants. <i>In vivo</i> injections of tritiated leucine into the blood of guinea pigs resulted in an incorporation of the amino acid to α_1-microglobulin in a subcellular subfraction of the liver. The radioactive protein was then released to the blood. Immunoregulatory properties of human α_1-microglobulin was shown. Antigen stimulation of peripheral blood lymphocytes <i>in vitro</i> was partly suppressed by α_1-microglobulin. Also, leukocyte migration was inhibited. Peripheral blood lymphocytes and some lymphoid cell-lines did not express α_1-microglobulin on their surfaces. The protein could not be detected in the culture medium of these cells. Thus, α_1-microglobulin is apparently not a lymphokine, but a non-specific immunosuppressive factor, synthesized by the liver.	
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Date November 8, 1982

α_1 -microglobulin

Studies in guinea pig and man

Bo Åkerström

α1-microglobulin

Studies in human and mouse

By J. Wahren

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Printed in Sweden

Studentlitteratur

Lund 1982

... Jody fell out of his tractor,
couldn't believe what he'd seen ...

John Fogerty (1969)



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LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. Bo Åkerström and Ingemar Berggård. Guinea pig α_1 -microglobulin. Isolation and properties in comparison with human α_1 -microglobulin. *Eur. J. Biochem.* 101, 215-223 (1979)
- II. Bo Åkerström. Tissue distribution of guinea pig α_1 -microglobulin. Submitted for publication.
- III. Bo Åkerström. Synthesis of α_1 -microglobulin by guinea pig liver. Submitted for publication.
- IV. Bo Åkerström, Kenneth Nilsson, Barbro Berggård and Ingemar Berggård. In search of α_1 -microglobulin on the lymphocyte surface. *J. Immunol.* 122, 2516-2520 (1979)
- V. Lennart Lögdberg and Bo Åkerström. Immunosuppressive properties of α_1 -microglobulin. *Scand. J. Immunol.* 13, 383-390 (1981)

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ABBREVIATIONS

α_1 -m	- α_1 -microglobulin
α_1 -MGP	- α_1 -microglycoprotein
Con-A	- Concanavalin-A
DEAE	- Diethylaminoethyl
HLA	- Human leukocyte antigen
L-chains	- Light chains of immunoglobulins
LNT	- Lymph node T-cells
PEC	- Peritoneal excudate cells
PEL	- Peritoneal excudate lymphocytes
PHA	- Phytohaemagglutinine
PPD	- Purified protein derivative of tuberculus bacteria
Protein HC	- Human complex-forming protein, heterogeneous in charge
RBP	- Retinol binding protein
SDS-PAGE	- Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis

INTRODUCTION

In the 1960's Ingemar Berggård and co-workers spent considerable time and effort investigating the protein fractions of human urine. With a few exceptions the urinary proteins originate from blood. It is generally accepted that the mechanism for excretion of plasma protein is glomerular filtration followed by reabsorption in the proximal tubules (*cf.* Strober and Waldmann, 1974). Methods were developed for differentiation of normal, glomerular, and tubular proteinuria. Normally, only low molecular weight plasma proteins are allowed to pass the glomerular barrier and are almost completely reabsorbed in tubuli. In glomerular disorders, there is an increased urinary excretion of relatively high molecular weight plasma proteins, since these are leaking through the malfunctioning glomerular membranes. In tubular dysfunctions, mostly low molecular weight plasma proteins are excreted in the urine. These are preferentially affected by the reduced reabsorption capacity of the tubules (*cf.* Berggård, 1970). Figure 1 (Peterson *et al.*, 1969) illustrates the difference. It shows the results from a gel-chromatographic separation of concentrated urine from patients with tubular (A), and glomerular (B) proteinuria.

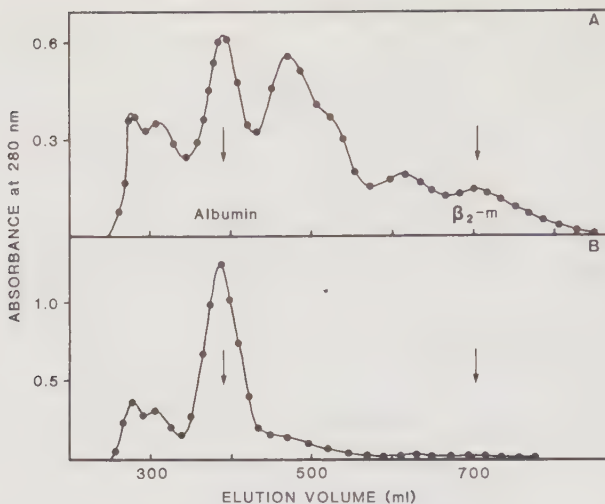


Figure 1. Gel chromatography on Sephadex G-100 of concentrated urinary proteins from a patient with chronic cadmium poisoning (A) and from a patient with glomerulonephritis (B). The content of total protein in the effluent was measured by absorbance at 280. The distribution of albumin and β_2 -microglobulin was determined by single radial immunodiffusion. (Peterson *et al.*, 1979).

Apparently, urine from patients with tubular malfunctions is a good starting material for the isolation and examination of small plasma proteins. In the 1960's and the beginning of the 1970's, Berggård isolated and characterized several protein components from urine. Figure 2 illustrates a gel-chromatography on Sephadex G-100 of a 24-hour volume of urine from a patient with tubular proteinuria (Cigén and Åkerström, unpublished data). Figure 2B shows the amount in the fractions of some specific proteins. Peak no. 1 contains mostly albumin. The second peak contains the dimer of the light chain of immunoglobulins (Berggård and Edelman, 1963). In the third peak is found a protein which Berggård first called "long- α_2 -globulin" (Berggård, 1961) but which was identified as the retinol-binding protein (RBP) (Peterson and Berggård, 1971). Peak no. 4 is β_2 -microglobulin (Berggård and Bearn, 1968), a protein later shown to be of considerable biological significance. The immunoglobulin light chains, the RBP, and β_2 -microglobulin are

present in blood in trace-amounts, but are apparently major constituents of urine from patients with tubular proteinuria.

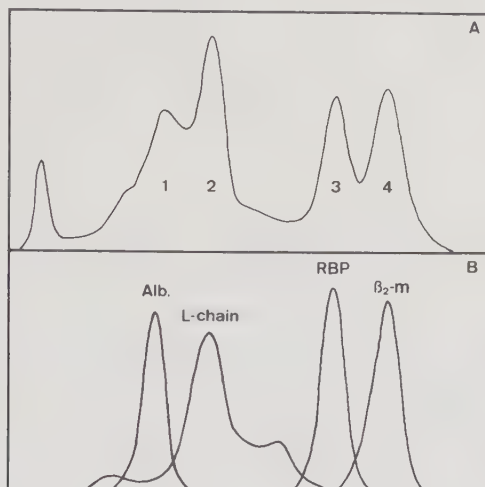


Figure 2. Gel chromatography on Sephadex G-100 of a concentrated 24-hour volume urine from one patient with tubular proteinuria. The column (3.5 x 130 cm) was eluted with 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.02% NaN₃ at 4°C. The flow-rate was 9.3ml/hour. Total protein in the effluent was measured as optical density at 280 nm (A). The concentration of albumin, L-chains, RBP and β_2 -microglobulin were determined by single radial immunodiffusion (B).

However, the material in peak no. 2 had a yellow-brown color, which could be monitored in the effluent from the gel-chromatography by the light-absorbance at 330 nm. The fact that the purified immunoglobulin light chains were completely without color, led to the conclusion that another molecule, carrying the brown color, was present in those fractions. The substance was isolated and characterized by Ekström *et al.* (1975), and is a glycoprotein with a molecular weight of 26,000 daltons, a heterogeneous net charge, and a brown color. It was named α_1 -microglobulin.

GENERAL KNOWLEDGE OF α_1 -MICROGLOBULIN

Since the first report on the isolation of α_1 -microglobulin was published from Lund by Ekström *et al.* (1975), several other investigators have presented results from studies on the protein. The groups are listed in Table I by their first publication on α_1 -microglobulin. Two other names for the protein have been proposed. Tejler and Grubb (1976) used the name protein HC, human complex-forming protein, heterogeneous in charge, and Seon and Pressman (1978) called it α_1 -MGP, α_1 -microglycoprotein. These two proteins and α_1 -microglobulin are biochemically very similar, and an immunological identity has been shown between protein HC and α_1 -microglobulin (Åkerström *et al.*, 1979: paper IV). Therefore, the name α_1 -microglobulin will be used throughout this presentation.

TABLE I

Different groups contributing to the research on α_1 -microglobulin

1st publication	Year	City, Country	Name of protein
Ekström <i>et al.</i>	1975	Lund, Sweden	α_1 -microglobulin
Tejler and Grubb	1976	Malmö, Sweden	protein HC
Svensson and Ravnskov	1976	Lund, Sweden	α_1 -microglobulin
Seon and Pressman	1978	Buffalo, USA	α_1 -microglycoprotein
Takagi <i>et al.</i>	1979	Tokyo, Japan	α_1 -microglobulin
Dautigny <i>et al.</i>	1979	Paris, France	α_1 -microglobulin
Vincent <i>et al.</i>	1982	Lyon, France	α_1 -microglobulin

Purification

In the first publication on α_1 -microglobulin, urine of patients with tubular proteinuria, caused by chronic cadmium poisoning, was used as starting material. The isolation procedure included four steps and is summarized in Table II. A comparison between various purification schedules, reported from the other groups, is also given in Table II. All investigators have used urine as the source of the protein. Tejler and Grubb (1976)

originally used normal urine, containing 10 mg α_1 -microglobulin/liter, for their purification, but later employed urine from a patient with mixed glomerular-tubular proteinuria (200 mg/l). Svensson and Ravnskov (1976) used urine from uraemic patients (49 mg/24-hour volume), Seon and Pressman (1978) urine from patients with plasma cell leukemia (140 mg/l), Takagi *et al.* (1979) and Dautigny *et al.* (1979) urine from patients with tubular proteinuria.

All groups have used the purified α_1 -microglobulin to raise antibodies against the protein, in one case monoclonal antibodies (Vincent *et al.*, 1982). These have been used as tools for the detection and quantitation of α_1 -microglobulin during the isolation, or analysis of the purified product. Thus, the α_1 -microglobulin of Ekström and Berggård (1977) appeared pure when tested with immunoelectrophoresis, immunodiffusion, sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and analysis of the amino-terminal amino acid sequence (Åkerström *et al.*, 1979: paper IV).

TABLE II

Purification of α_1 -microglobulin by different investigators

	Ekström <i>et al.</i> (1975)	Tejler and Grubb (1976)	Svensson and Ravnskov (1976) Takagi <i>et al.</i> (1979)	Seon and Pressman (1978)	Bernier <i>et al.</i> (1980)	Vincent <i>et al.</i> (1982)
Starting material	Concentrated urine					
Step 1	Sephadex G-100	DEAE-Sephadex pH 8.0	Con-A-Sepharose	Ammonium-sulfate precipitation 65%	DEAE-Sephadex pH 8.0	Sephadex G-100
Step 2	Zone-electro- phoresis pH 8.6	Ultrogel AcA 54	Sephadex G-75	Sephadex G-200	Sephadex G-150	Zone-electro- phoresis pH 8.6
Step 3	Sephadex G-100	Ultrogel AcA 54	Whatman DE 52 pH 7.85	DEAE-cellulose pH 8.0	Electrofocusing pH 3.5 - 5.0	Sephadex G-100
Step 4	DEAE-Sephadex pH 7.5	Ultrogel AcA 54		DEAE-cellulose pH 8.0		DEAE-Sephadex pH 7.5
Step 5		Anti-albumin Sepharose		Sephadex G-100		Con-A-Sepharose

The investigators have purified a seemingly identical product, as judged by biochemical criteria. However, contradictory data have been reported concerning the distribution of α_1 -microglobulin in tissues (see below), and in some cases the discrepancies could be attributed to the presence of impurities in the preparations of α_1 -microglobulin (Åkerström *et al.*, 1979: paper IV, Dautigny *et al.*, 1979). Since a highly purified and well-defined antigen is essential for non-competitive immunological techniques such as immunofluorescence, even using monoclonal antibodies, we have modified the original purification procedure of α_1 -microglobulin (Lögberg and Åkerström, 1981: paper V).

Biochemical properties

Size and shape

Ekström and Berggård (1977) have made a detailed physicochemical description of α_1 -microglobulin. Some of the properties are listed in Table IV. The molecular weight of about 26,000 has been estimated by ultracentrifugation and determination of Stokes' radius, and by gel-chromatography in 6M guanidine-HCl (Ekström and Berggård, 1977, Seon and Pressman, 1978, Bernier *et al.*, 1980). SDS-PAGE (Figure 3A) gives the apparent molecular weight of 31,000 (Tejler and Grubb, 1976, Svensson and Ravnskov, 1976, Ekström and Berggård, 1977, Seon and Pressman, 1978, Takagi *et al.*, 1979, Bernier *et al.*, 1980, Vincent *et al.*, 1982). The overestimation of the molecular weight by this method is probably due to the fact that α_1 -microglobulin contains carbohydrate and that it has an elongated shape or a high degree of hydration, which is suggested by the relatively high frictional ratio, 1.45. A practical consequence of this property is that on gel-chromatography, α_1 -microglobulin is co-eluted with macromolecules of higher molecular weight than its own. Hence, impurities in α_1 -microglobulin preparations can be expected among urinary proteins with a relatively high molecular weight.

Purified α_1 -microglobulin has a tendency to form dimers in water solutions, as it is eluted as two peaks on gel-chromatography (Ekström and Berggård, 1977). Apparently the monomers do not form covalently linked dimers, since only one band, corresponding to monomeric α_1 -microglobulin, can be detected when submitting the dimer from a gel-chromatography to SDS-PAGE without reducing agents (Ekström and Berggård, 1977).

The presence of an intra-chain disulfide bond and a free cysteine molecule, involved in an S-S bond with a half-cystine residue, was suggested by Åkerström and Berggård (1979: paper I) and by Mendez *et al.* (1982). The intra-chain disulfide bridge was confirmed by sequence analysis of the protein (Mendez *et al.*, 1982).

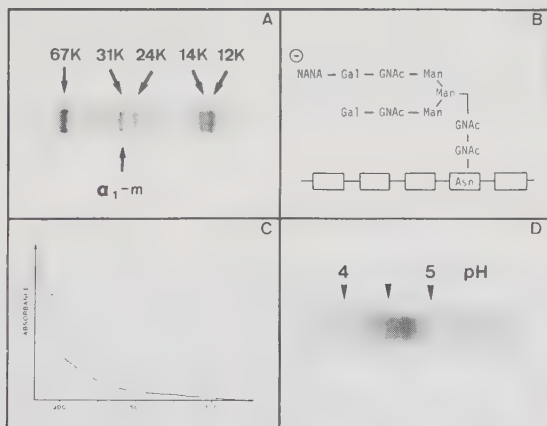


Figure 3. Some physicochemical parameters of α_1 -microglobulin. **A.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of α_1 -microglobulin. The gel was run from left to right. Human serum albumin, immunoglobulin L-chains, egg white lysozyme and guinea pig β_2 -microglobulin were used as markers of molecular weights. **B.** Structure of oligosaccharide of human α_1 -microglobulin (Ekström *et al.*, 1981). **C.** Absorbance spectrum of guinea pig α_1 -microglobulin in 0.1 M phosphate buffer pH 7.5. **D.** Isoelectric focusing of human α_1 -microglobulin, on a polyacrylamide gel with Ampholines between pH 3.5-9 (Åkerström and Berggård, 1979).

Carbohydrate

The carbohydrate portion, representing approximately 20% of the total weight of α_1 -microglobulin, has been subjected to structure analysis (Ekström *et al.*, 1981). It was found that the monosaccharides sialic acid, galactose, mannose, and glucosamine, previously found in the protein (Ekström and Berggård, 1977), were arranged in three oligosaccharides, N-glycosidically linked to asparagine residues in the polypeptide chain. The sequence was determined by methylation analysis in combination with specific chemical degradation techniques. Figure 3B shows the oligosaccharide. It has not been determined which branch the sialic acid residue is bound to, nor if it is bound to both branches. The occurrence of carbohydrate linked to asparagine residues in the peptide chain through an N-glycosidic bond, involving N-acetyl glucosamine, has been observed in a number of glycoproteins from diverse sources. However, one O-glycosidically linked oligosaccharide at amino acid residue no. 5 (threonine) has been suggested by Takagi *et al.* (1981) and Lopez *et al.* (1981), from results obtained by amino acid sequence analysis.

Color

It has been mentioned earlier that a solution of α_1 -microglobulin, as well as the freeze-dried protein, has a yellow-brown color. The color is visible in solutions from about 0.1 mg/ml, and at 50 mg/ml, the solution has an intense dark-brown, almost black color. This property is unique among the urinary proteins investigated so far. α_1 -microglobulin, purified from human plasma (Åkerström, unpublished work), is also colored in this way. The absorption spectrum of α_1 -microglobulin (Figure 3C) shows a plateau starting at approximately 300 nm, and is decreasing slowly throughout the visible region. This indicates the presence of a chromogeneous constituent other than amino acid or carbohydrate. Attempts to dissociate the colored substance from the apo-protein have been made by different means. Treatment with acid, 6M guanidine-HCl, SDS, neuraminidase, and reduction and alkylation have been unsuccessful

(Tejler and Grubb, 1976, Ekström and Berggård, 1977, Seon and Pressman, 1978). This indicates that the chromophore is covalently linked to the protein. The absence of any absorption maxima, except for that at 278 nm, suggests that this substance is not homogeneous. By adding the molecular weight of the amino acid residues of α_1 -microglobulin (20,435, Lopez *et al.*, 1981) and the weight of 3 oligosaccharides of the type shown in Figure 3B (5,739, Ekström *et al.*, 1981), the calculated molecular weight of α_1 -microglobulin, without the chromophore, is 26,174. The agreement between the hydrodynamically measured molecular weight (26,000), and the above calculated molecular weight suggests that the brown-colored material is indeed a small molecule. However, Lopez *et al.* (1981) propose several points of attachment for the chromophore on the polypeptide, since all but one of the CNBr-fragments were colored (Grubb, personal communication). This is supported by findings in our laboratory. On the contrary, Takagi *et al.* (1981) suggest that the chromophore is bound to one of the asparagine-linked carbohydrate moieties. They do not, however, present data to support their proposition.

Charge

The charge-heterogeneity of α_1 -microglobulin has been observed by all investigators. The protein migrates in the α_1 -region as a broad zone on agarose gel-electrophoresis, and several subpopulations with different isoelectric points can be separated by isoelectric focusing on polyacrylamide gels, as shown in Figure 3D. The range of pI-values for α_1 -microglobulin purified from pathological urine is 4.3-4.8 (Seon and Pressman, 1978, Åkerström and Berggård, 1979: paper I, Bernier *et al.*, 1980, Vincent *et al.*, 1982), while α_1 -microglobulin from normal urine seems to be more acidic (Tejler and Grubb, 1976, Bernier *et al.*, 1980). Bernier *et al.* (1980) reported small differences in amino acid composition of α_1 -microglobulin from normal and pathological urines, but no variation within either type. In our hands, there seems to be no variation between α_1 -microglobulin purified from different individuals, since isoelectric focusing pattern of the protein was identical, regardless of the source.

(Åkerström *et al.*, 1979: paper I). The amino acid sequence of α_1 -microglobulin in urine from a single individual (Lopez *et al.*, 1981), or in pooled urine (Takagi *et al.*, 1981), was homogeneous. Neuraminidase treatment of the protein did not decrease the charge heterogeneity, although the net charge was reduced (Ekström *et al.*, 1975, Tejler and Grubb, 1976, Bernier *et al.*, 1980, Vincent *et al.*, 1982). Hence, the charge heterogeneity seems to reside in the material responsible for the brown color (see also below).

Amino acid sequence

The amino acid sequence of α_1 -microglobulin has been determined by Takagi *et al.* (1981) and Lopez *et al.* (1981). The cysteine residue at positions 70 and 167 form an intra-chain disulfide bridge, and cysteine at position 34 seems to be involved in an S-S bond with free cysteine or cysteine-containing peptides (Mendez *et al.*, 1982). It is suggested that part of the heterogeneity of α_1 -microglobulin is explained by a variation of the substances bound to cysteine at position 34.

Distribution of α_1 -microglobulin

Plasma

The presence of α_1 -microglobulin in human plasma has been demonstrated by immunological analysis. Ekström *et al.* (1975) showed that the material reacting with anti- α_1 -microglobulin was eluted as four components of different size, when human plasma was chromatographed on a column of Sephadex G-200 (Figure 4). The last eluted peak corresponds in size to α_1 -microglobulin isolated from urine. Tejler and Grubb (1976) showed that α_1 -microglobulin in human plasma forms complexes with IgA and albumin. These are probable candidates for the second and third α_1 -microglobulin containing peaks of the gel-chromatogram.

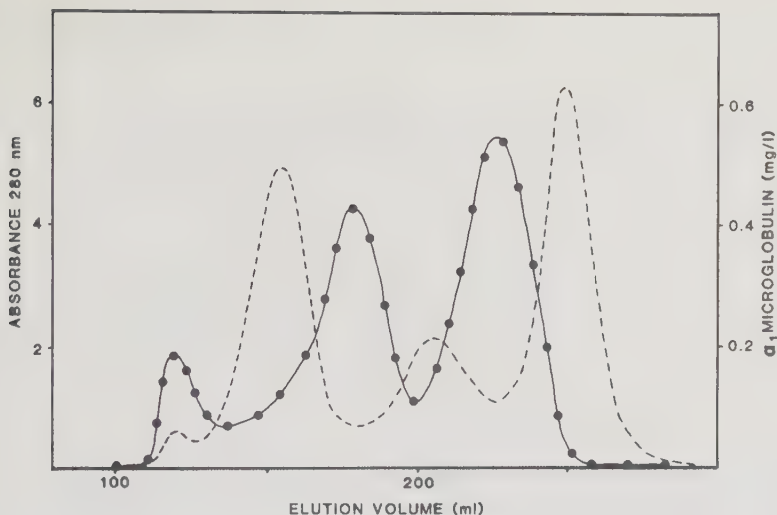


Figure 4. Gel chromatography on Sephadex G-200 of human serum. Total protein (●—●) was measured as absorbance at 280 nm, and α_1 -microglobulin (----) was determined by radioimmunoassay.

It is likely that the different α_1 -microglobulin-complexes are partly responsible for the discrepancies in the various reports on the normal plasma concentration of the protein. Thus, various estimations on the mean level of α_1 -microglobulin in normal human plasma range from 1.8 mg/l to 98 mg/l. (see Table III). It is difficult to explain these major discrepancies only in terms of the different forms of α_1 -microglobulin in plasma, since the size distribution of the protein has shown very little variation in gel-chromatography of normal plasma.

Experimental parameters, such as choice of method and standard α_1 -microglobulin, dilution of the test-sample, are naturally of importance.

TABLE III

Concentration of α_1 -microglobulin in normal human plasma, reported by different investigators.

Publication	α_1 -microglobulin (mg/l)	Method
Tejler and Grubb (1976)	98	Electroimmunoassay
Svensson and Ravnskov (1976)	32	Electroimmunoassay
Ekström and Berggård (1977)	54	Single radial immunodiffusion
Takagi <i>et al.</i> (1980a)	9	Enzyme-linked immunoassay
Takagi <i>et al.</i> (1980b)	44	Single radial immunodiffusion
Itoh (1981)	1.8	Single radial immunodiffusion

A comparison of α_1 -microglobulin levels in body fluids, as reported by different investigators, shows contradictory results. Only in patients with renal disorders have elevated plasma-concentrations of α_1 -microglobulin been found by more than one author (Ekström *et al.*, 1975, Tejler and Grubb, 1976, Svensson and Ravnskov, 1976, Takagi *et al.*, 1980a, Takagi *et al.*, 1980b). Lower levels of the protein in females than in males have been reported by Tejler (1978), and Takagi *et al.* (1980b). An interesting observation was made by the latter group, where the difference between the plasma levels of males and females was greatest during the first few years, and negligible during the last years of life. The contradictory results concerning concentrations of plasma α_1 -microglobulin in patients with liver cirrhosis will be discussed in the next section.

THE PRESENT INVESTIGATION

The two main themes of this thesis are the introduction and use of an animal homologue in the α_1 -microglobulin research, and an investigation of a possible immunological role for α_1 -microglobulin.

Purification of guinea pig α_1 -microglobulin (I)

Urine is an excellent source for the purification of α_1 -microglobulin from humans, especially from patients with tubular proteinuria. Maleic acid (Berliner *et al.*, 1950) and sodium chromate (Balazs and Roepke, 1966) have been shown to cause tubular malfunctions in animal kidneys. Both of these nephrotoxic agents were tried on guinea pigs (Berggård and Berggård, unpublished work). As maleic acid was found to induce a less prominent tubular proteinuria than sodium chromate, the latter reagent was used for the isolation of α_1 -microglobulin from guinea pigs. Sodium chromate has also been shown to give rise to microglobulinuria in the rabbit (Berggård, 1974) and in the rat (Lögdberg *et al.*, 1979).

The isolation procedure for guinea pig α_1 -microglobulin was essentially the same as the purification of the human protein. During the original procedure, the absorbance of light at 330 nm was used to trace α_1 -microglobulin in the urine fractions. Also, the brown color made it possible to follow the protein visually. The purified product was used to prepare antisera from rabbits, and with these, α_1 -microglobulin could be determined immunochemically in later purifications. The purification steps included gel-chromatographies, zone-electrophoresis and ion-exchange chromatography, and are illustrated in Figure 5. α_1 -microglobulin comprised 3-6% of the proteins in the starting material, and the yield was 10-15%. In a representative experiment, 32% of applied α_1 -microglobulin was lost during zone-electrophoresis (Figure 5B), mostly due to the charge-heterogeneity of the protein, and 50% was lost during each gel-chromatography (Figure 5A and 5C), mainly because of

dimerization of the protein.

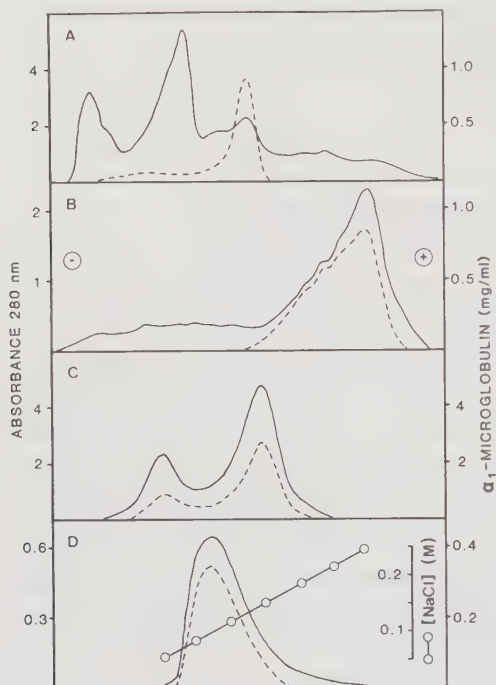


Figure 5. (Åkerström and Berggård, 1979, paper I). Purification of guinea pig α_1 -microglobulin. Total protein (—) was determined by absorbance at 280 nm, and α_1 -microglobulin (----) was measured by single radial immunodiffusion. A. Gel chromatography on Sephadex G-100 of concentrated urine from guinea pigs treated with sodium chromate. The column was eluted with 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.02% NaN₃. B. Zone electrophoresis of pooled α_1 -microglobulin-containing fractions from 5a. Pevikon C-870 was used as supporting material, and the electrophoresis was done in 0.1 M sodium-borate buffer, pH 8.9. C. Gel chromatography on Sephadex G-100 of pooled α_1 -microglobulin-containing fractions from 5b. D. Ion-exchange-chromatography on DEAE-cellulose of pooled α_1 -microglobulin-containing fractions from 5c. The column was equilibrated with 20 mM Tris-HCl, pH 8.0 and 50 mM NaCl. It was eluted with a linear gradient from 0.05 M to 0.30 M, and the sodium chloride concentration in the effluent was followed by the conductivity.

We have made preliminary purifications of α_1 -microglobulin from rabbits and rats (Åkerström, unpublished work), using urine from animals with sodium chromate-induced proteinuria. Also, from these species, a glycoprotein, associated with a brown color, a tendency to form dimers, and a charge heterogeneity, was purified.

Physicochemical properties of guinea pig α_1 -microglobulin (I)

A homology between guinea pig and human α_1 -microglobulin could be demonstrated by a close biochemical resemblance and an immunological cross-reaction. Some physicochemical parameters of guinea pig and human α_1 -microglobulin are listed in Table IV. The charge of guinea pig α_1 -microglobulin from different

preparations, and of human α_1 -microglobulin from different individuals was compared by isoelectric focusing. All of them showed an identical pattern (Figure 3D).

TABLE IV

Physical and chemical properties of α_1 -microglobulin

Parameter	Human ^{A)}	Guinea pig ^{B)}
Sedimentation coefficient, $s_{20,w}^0$	2.35	2.46 S
Stokes' molecular radius, r_s	28.5	25.2 Å
Partial specific volume, $\bar{v}$	0.704	0.709 ml/g
Apparent diffusion coefficient, $D_{20,w}^0$	75	85 $\mu\text{m}^2\text{s}^{-1}$
Frictional ratio, f/f_0	1.45	1.34
Molecular weight		
By sedimentation equilibrium ultracentrifugation	26,700	
From sedimentation and diffusion coefficients	26,100	24,000
By gel-chromatography in 6 M guanidine-HCl, reduction and alkylation	24,800	23,000
By electrophoresis in SDS	31,000	25,800
Absorption coefficient at 280 nm,	4.72	$5.14 \cdot 10^4 \text{ M}^{-1}\text{cm}^{-1}$
Free sulfhydryl groups	Nil	Nil
pI ^{B)}	4.3 - 4.8	4.3 - 4.8

A) Ekström and Berggård, 1977.

B) Åkerström and Berggård, 1979.

The molecular weight of guinea pig α_1 -microglobulin, 23,500 is smaller than the molecular weight of human α_1 -microglobulin, 26,000 (Ekström and Berggård, 1977). Thus, we investigated the presence of a larger component in the urine from guinea pigs by immunoprecipitation with anti-guinea pig α_1 -microglobulin of radiolabelled proteins from gel-chromatography fractions where such a component could be expected. No larger form of guinea pig α_1 -microglobulin was found. The sedimentation coefficient, Stokes' radius, diffusion coefficient, and frictional ratio, were similar to corresponding data for human α_1 -microglobulin, and suggest an elongated shape or/and a high degree of hydration.

The amino acid and carbohydrate composition of human and guinea pig α_1 -microglobulin are shown in Table V. The values for guinea pig α_1 -microglobulin are based on the assumption that the total weight of the amino acids amounts to 84% of the molecular weight for guinea pig α_1 -microglobulin (23,500). The amino acid composition of the two homologues are very similar, except for isoleucine, of which the guinea pig homologue contains 7 residues and human α_1 -microglobulin 13 residues. The amino acid composition of rabbit and rat α_1 -microglobulin (Åkerström, unpublished work), are included in Table V, and illustrate the homology between α_1 -microglobulin from the four mammalian species investigated so far. An intra-chain disulfide bridge was found in guinea pig α_1 -microglobulin, as concluded from the faster migration of the non-reduced compared to the reduced (mercaptoethanol) protein upon SDS-PAGE. After dialysis of reduced and alkylated guinea pig α_1 -microglobulin, the number of half-cystine residues was determined to 3.2, and 4.4 residues were found in the non-treated protein. This is best interpreted as the presence of one cysteine residue, involved in an S-S bond with a free, dialyzable cystein molecule, and is in agreement with the proposed structure for human α_1 -microglobulin (Mendez *et al.*, 1982).

Tissue distribution of α_1 -microglobulin (I, II, IV)

Serum and urine (I)

The concentration of α_1 -microglobulin in normal guinea pig serum was determined to 26.4 mg/l by radioimmunoassay. The α_1 -microglobulin in serum was immunologically identical to the protein purified from urine, when tested on Ouchterlony immunodiffusion. Gel-chromatography of guinea pig serum de-

monstrated four different sizes of the material reacting with anti- α_1 -microglobulin. The behavior was similar to α_1 -microglobulin in human serum (Figure 4). α_1 -microglobulin in urine from normal guinea pigs and guinea pigs treated with sodium chromate amounted to 1.4 mg and 4.6 mg/24-hour volume, respectively.

TABLE V

Amino acid and carbohydrate composition of human, guinea pig, rabbit and rat α_1 -microglobulin

Residue	Human		Guinea ^{C)} pig	Rabbit ^{D)}	Rat ^{D)}
	A)	B)			
<i>Amino acids</i>					
Aspartic acid/asparagine	14	14.3	15.8	14.7	17.6
Threonine	17	16.9	15.6	13.1	12.1
Serine	10	10.3	12.4	10.7	12.2
Glutamic acid/glutamine	21	21.7	22.1	25.5	26.1
Proline	12	13.5	10.8	11.2	8.7
Glycine	14	14.0	13.5	11.3	12.6
Alanine	9	9.3	9.7	12.3	10.2
Cysteine	3	2.3	3.2		
Valine	11	10.9	8.4	13.0	14.0
Methionine	5	4.5	4.7	2.5	1.5
Isoleucine	13	12.4	7.0	7.6	7.8
Leucine	11	11.0	13.3	14.3	12.4
Tyrosine	8	8.3	6.9	5.9	5.6
Phenylalanine	6	6.2	5.0	5.6	6.6
Lysine	10	10.6	9.3	10.2	9.7
Histidine	4	3.6	3.8	2.8	3.5
Arginine	10	9.7	10.6	8.0	8.3
Tryptophan	3	3.7	3.3		
<i>Carbohydrates (%)</i>					
Hexose		6.4	7.7		
Glucosamine		5.1	3.8		
Sialic acid		5.5	4.2	4.4	

A) Lopez *et al.* (1981). Based on the amino acid sequence.

B) Ekström and Berggård (1977). Calculated on the basis of 11.0 residues of leucine per molecule.

C) Akerström and Berggård (1979). Based on a molecular weight of 23,500.

D) Bo Akerström, unpublished. Calculated on the basis of the same total amount of amino acid residues per molecule as guinea pig α_1 -microglobulin.

Two groups have demonstrated the binding of anti- α_1 -microglobulin to the surface of human erythrocytes and various lymphoid cells and cell-lines (Tejler *et al.*, 1976, Pearlstein *et al.*, 1977, and Takagi *et al.*, 1979, Itoh *et al.*, 1979). Both groups used immunofluorescence to show the binding.

In paper IV, we report that there was no binding to established lymphoid cell-lines of anti- α_1 -microglobulin, when tested with immunofluorescence. A weak immunofluorescence was demonstrated on normal peripheral lymphocytes, but could not be inhibited by purified α_1 -microglobulin. Radioimmunoassay of normal blood lymphocytes, erythrocytes and two lymphoid cell-lines, solubilized with various agents, was completely negative with respect to α_1 -microglobulin. Parallel experiments with anti- α_1 -microglobulin from Tejler and Grubb gave positive immunofluorescence for most of the cell-lines and for normal lymphocytes. α_1 -microglobulin purified by Tejler and Grubb could not block the fluorescence. This suggests a non-specific staining of the cells. We could not find any measurable amounts of α_1 -microglobulin in the culture medium of lymphoid cell-lines or peripheral white blood-cells stimulated with phytohaemagglutinine (PHA).

Moreover, α_1 -microglobulin was not released from peripheral lymphocytes or erythrocytes of the guinea pig, when solubilizing these cells in different ways (paper II).

Cross-examination of the two α_1 -microglobulin preparations and the corresponding antisera, using ^{125}I -labeling, immunoprecipitation and SDS-PAGE, revealed high molecular weight contaminants in both preparations. α_1 -acid glycoprotein, a lymphocyte-membrane protein (Gahmberg and Andersson, 1978), was present in the α_1 -microglobulin sample from Tejler and Grubb.

We therefore believe that the membrane fluorescence detected by Tejler *et al.* and Takagi *et al.* is due to at least one contaminant in the α_1 -microglobulin preparations, and that α_1 -microglobulin is not a membrane protein of lymphocytes.

Since the publication of paper IV, the absence of α_1 -microglobulin on the lymphocyte surface has been confirmed by a French group, Dautigny *et al.* (1979), who failed to stain human peripheral blood lymphocytes or Raji and Daudi cell-lines with fluorescein-coupled anti- α_1 -microglobulin. This group had earlier reported purification from urine of a protein with HLA-activity, presumed to be HLA (Bernier *et al.*, 1977) until sequence analysis of the amino-terminus showed identity with α_1 -microglobulin (Bernier *et al.*, 1978). The isolated α_1 -microglobulin was contaminated with 4% HLA (Dautigny *et al.*, 1979).

Vincent *et al.* (1982), recently demonstrated binding of anti- α_1 -microglobulin antibodies to human white blood cells and some cell-lines, among them Daudi and Raji. The anti- α_1 -microglobulin antibodies were monoclonal, and therefore monospecific. However, the molecule bound by the antibodies has not been properly characterized as α_1 -microglobulin. If the antibodies are directed against a determinant on α_1 -microglobulin, this may be part of the carbohydrate, a structure which could be distributed on a variety of plasma proteins and, probably, cell surfaces. Another, and interesting possibility is that the monoclonal antibodies recognize the chromophore. If so, is this substance bound to other molecules than α_1 -microglobulin on the cell surface of blood cells?

Other cells and tissues (II)

Figure 6A shows the distribution of guinea pig α_1 -microglobulin detected by radioimmunoassay, in various tissues, solubilized by mechanical (sonication) or chemical (deoxycholate) means. The bars represent the ratio α_1 -microglobulin $\times 10^3$ to albumin, rather than the absolute concentration of α_1 -microglobulin, in order to circumvent the problem with contamination by serum.

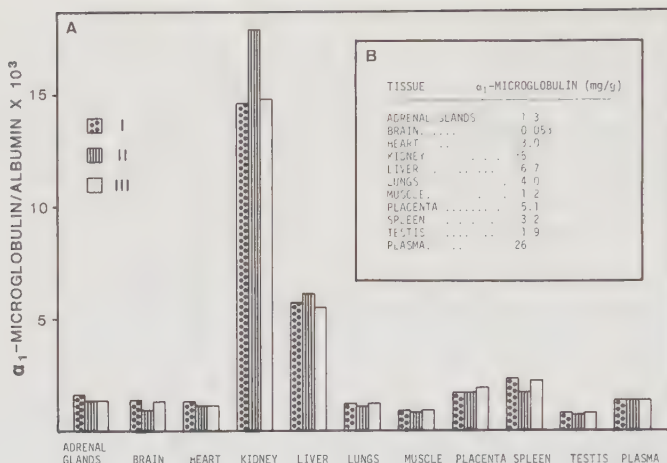


Figure 6. Tissue distribution of guinea pig α_1 -microglobulin, represented by the ratio of the concentrations of α_1 -microglobulin and albumin. (A) The tissues were homogenized (I), homogenized and solubilized with 0.5% deoxycholate (II), or homogenized and sonicated for 2×10 seconds (III). α_1 -microglobulin was measured by radioimmunoassay and albumin was determined by single radial immunodiffusion. (B) Absolute concentrations of α_1 -microglobulin in the tissues. The values are means of determinations in I, II and III.

The adrenal glands, brain, heart, lungs, muscle, placenta, spleen, and testes represent a background level of the ratio, not differing from serum. The α_1 -microglobulin detected in these organs is probably α_1 -microglobulin from remaining blood. Higher values were obtained for the liver and the kidney. The absolute

figures of α_1 -microglobulin ($\mu\text{g/g}$ tissue) are shown in Figure 6B. α_1 -microglobulin concentrations in the kidney are higher than in plasma. This organ has been shown to be the catabolic site of many low molecular weight plasma proteins (*cf.* Strober and Waldmann, 1974), which is a probable explanation for the high α_1 -microglobulin level in this organ. Likewise, it is tempting to explain the relatively high α_1 -microglobulin/albumin ratio in the liver as being due to a synthesis of α_1 -microglobulin in this organ.

Synthesis of α_1 -microglobulin (II, III)

With the isolation of guinea pig α_1 -microglobulin, we were provided with a tool for the examination of α_1 -microglobulin synthesis in guinea pigs, obviously methodologically preferable to studies in human organs. Thus, it was found that suspensions of guinea pig liver cells released small amounts of α_1 -microglobulin into the medium (paper II). The release was inhibited by sodium azide, indicating that it was not due to passively absorbed α_1 -microglobulin from blood.

Paper III demonstrated the synthesis of α_1 -microglobulin by guinea pig liver. [^3H]leucine was injected into the bloodstream of anesthetized guinea pigs, and after different time intervals samples were taken from the liver and blood. By immunoprecipitation with anti- α_1 -microglobulin it could be shown that [^3H]leucine was incorporated into α_1 -microglobulin in the 105,000 G pellet of the liver, reaching maximum values after 20 minutes. At that time, [^3H] α_1 -microglobulin was starting to accumulate in serum (Figure 7A). The kinetics of free [^3H]leucine in serum and of radioactive protein in 105,000 G pellet of liver and in serum, are shown in Figure 7B. Moreover, it was demonstrated that guinea pig liver tissue explants, cultured *in vitro* with [^3H]leucine, released [^3H] α_1 -microglobulin to the medium. α_1 -microglobulin was identified by immunoprecipitation and SDS-PAGE.

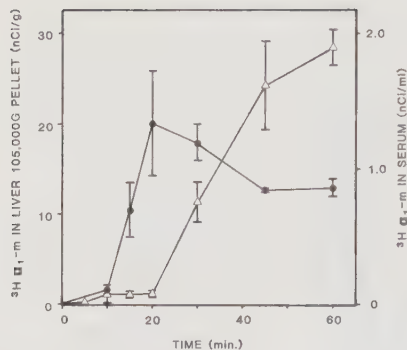


Figure 7A ^3H α_1 -microglobulin in liver 105,000 x G pellet (●—●), and serum (Δ—Δ), after injection of 0.5 mCi ^3H leucine/kg guinea pig. Each point represents the mean $\pm$ S.D. of two animals.

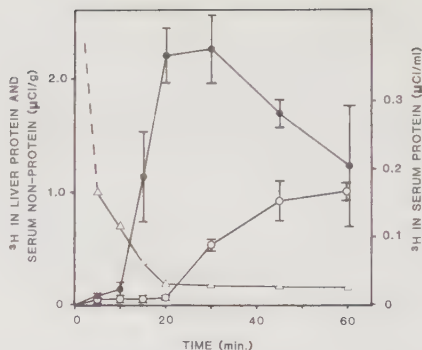


Figure 7B ^3H leucine in blood (Δ—Δ), liver protein (●—●), and blood protein (○—○), after injection of 0.5 mCi ^3H leucine/kg guinea pig. Each point represents the mean $\pm$ S.D. of two animals.

It is difficult to calculate the rate of synthesis from data such as these, since accurate determinations of the specific radioactivity of precursor-leucine are not readily obtained. However, the synthesis rate of albumin is known for humans and rats, and by comparing the kinetics of α_1 -microglobulin and albumin in serum, the synthesis of α_1 -microglobulin could be estimated to 20 ($\pm$ 8) $\mu\text{g/g}$ liver and hour.

Tejler *et al.* (1978) have shown that human fetal liver explants synthesize α_1 -microglobulin. This organ is also known to be a major site of hematopoiesis. Although the findings in paper III seem to connect the synthesis of α_1 -microglobulin with the hepatic rather than the hematopoietic function of the fetal liver, we still do not know which cell-type is responsible for the production of the protein. Apart from hepatocytes, endothelial vessel cells, bile duct cells and Kupfer cells can be found in the liver, and more work will have to be done to find the cell-types responsible for the production of α_1 -microglobulin.

Contradictory results have been published concerning the plasma concentrations of α_1 -microglobulin in patients with liver diseases. Thus, Svensson and Ravnskov (1976) reported normal values for five patients with liver cirrhosis, while Tejler (1978) showed subnormal concentrations in patients with various liver disorders, including liver cirrhosis. Various liver disorders, including liver cirrhosis, were associated with normal levels of α_1 -microglobulin, according to Takagi *et al.* (1980b), while Takagi *et al.* (1980a) demonstrated significantly decreased concentrations in 12 patients with liver cirrhosis. These results are difficult to discuss, and may serve as another example of the conflicting results that can be produced in the field of α_1 -microglobulin.

Immunoregulatory properties of α_1 -microglobulin (V)

Several immunosuppressive factors have been described in plasma (*cf.* Tomasi, 1977). Most of them belong to the α -globulin region of plasma proteins, and some of them are acute-phase reactants (*cf.* Koj, 1974). α_1 -microglobulin is probably not a lymphokine (paper IV), and according to Tejler (1978) and Takagi *et al.* (1980b), not an acute-phase reactant. It unquestionably is an α -globulin in plasma. In paper V, we have been able to show that human α_1 -microglobulin partly inhibits the proliferative response of peripheral blood lymphocytes to the antigens purified protein derivative (PPD) and tetanus toxoid. The lymphocyte response was measured as the incorporation of tritium-labeled thymidine by the cells. Studies of the dose-response relationships demonstrate a small degree of inhibition by normal plasma-concentrations of α_1 -microglobulin (0.03-0.1 mg/ml), and more prominent inhibitions at elevated plasma concentrations of α_1 -microglobulin. Hence, the increased plasma levels of α_1 -microglobulin in patients with renal failures (discussed above), could account for at least part of the impaired immune response associated with uremia (*cf.* Touraine *et al.*, 1975).

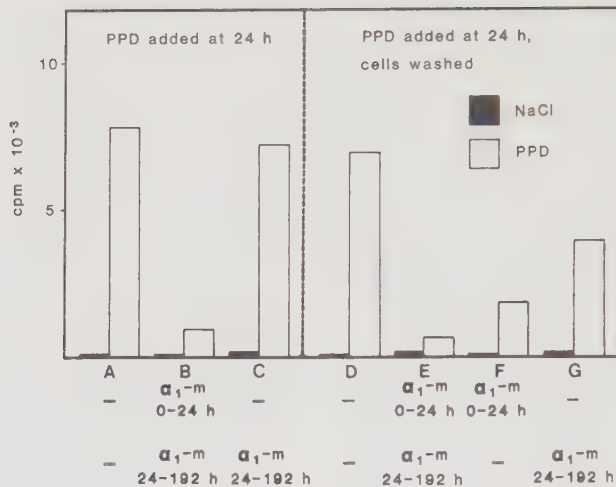


Figure 8. Effects of α_1 -microglobulin on antigen (PPD) stimulation of human lymphocytes after preculturing of the cells for 24 hours before the addition of PPD. The cells were washed (D-G), or not washed (A-C). The time-period during which α_1 -microglobulin was present, is indicated.

To investigate the stability of the suppressor mechanism, by which α_1 -microglobulin inhibits antigen-stimulations, a set of experiments were performed, where α_1 -microglobulin was either present, or not present, during a 24-hour preincubation of the cells without antigen (PPD) (Figure 8, Lögdberg and Åkerström, unpublished work). When α_1 -microglobulin was present during the preincubation, inhibitions were noted (Figure 8B,E,F), even if α_1 -microglobulin was totally removed by washing and not present during the antigen-stimulation (Figure 8F). However, if α_1 -microglobulin was not added until after the preincubation, no inhibition was noted (Figure 8C), indicating that α_1 -microglobulin acts through an unstable suppressor-mechanism, that it inactivated during the 24 hours of preculturing. However, once triggered by α_1 -microglobulin, the apparently cellular mechanism (Figure 8F) is able to exercise the suppression by itself.

Another *in vitro* result of antigen-stimulation of lymphocytes is the decreased migration of leukocytes under agarose. Surprisingly, α_1 -microglobulin did not affect this trait, but instead had its own leukocyte migration inhibitory effect. Unpublished observations (Lögdberg and Åkerström) suggest that α_1 -microglobulin exerts this inhibition via another lymphocyte population, possibly the same cell-type mediating the inhibition of antigen-stimulation.

Preliminary results (Lögdberg, Åkerström and Shevach) from studies with guinea pig lymph-node T-cells (LNT) and lymphocytes (PEL) from peritoneal exudate cells (PEC), show that α_1 -microglobulin is mitogenic to LNT and PEL, but only if these are supplemented with PEC. Furthermore, studies on human white blood-cells (Åkerström, Curtiss, and Edgington, unpublished work), suggest that low-affinity receptors for α_1 -microglobulin are expressed on monocytes and lymphocytes. These results make it tempting to construct a model for the interaction of α_1 -microglobulin with antigen-stimulation and leukocyte migration, such as shown in Figure 9.

It should be emphasized that the sequence of interactions in the figures are based on preliminary findings. Still remaining is to find the exact nature of the cell-types involved in physical interactions with α_1 -microglobulin, of the cell-types being stimulated by the protein, and of the cell-types enforcing the final effects on the antigen-stimulation and leukocyte migration. Such work is in progress.

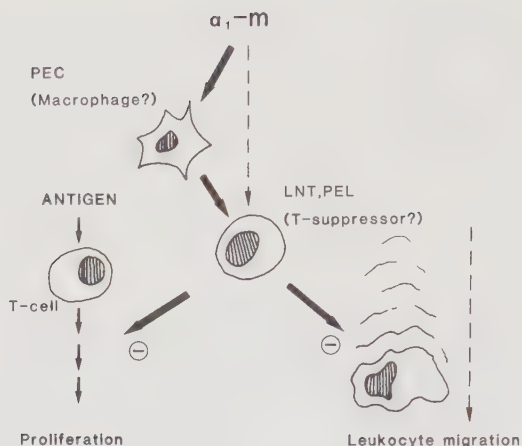


Figure 9. Tentative model for the immunosuppressive effects of α_1 -microglobulin. The inhibition of antigen-stimulation and leukocyte-migration, is mediated via a T-suppressor cell. Macrophages are needed, as accessory cells, for the activation of the T-suppressor cells. These require a signal from only the macrophages, or from both macrophages and α_1 -microglobulin.

CONCLUDING REMARKS

Guinea pig α_1 -microglobulin has been purified and characterized. A close biochemical resemblance, and an immunological cross-reaction with human α_1 -microglobulin was demonstrated. It was the first animal homologue of α_1 -microglobulin to be isolated, and it permitted an investigation of the protein in various guinea pig organs. Here, α_1 -microglobulin was localized preferentially to the liver and the kidneys. A *de novo* synthesis was demonstrated in liver explants. *In vivo*, the newly synthesized protein first appeared in a subcellular fraction of the liver, and was then released into the blood.

At a time when the literature on the synthesis of α_1 -microglobulin was contradictory, and lymphocytes were reported to produce the protein, we investigated the presence of α_1 -microglobulin in lymphocytes. No production or expression of α_1 -microglobulin from these cells was detected. However, α_1 -microglobulin possessed immunoregulatory properties. It was found to inhibit antigen-stimulation of lymphocytes, and also to inhibit leukocyte-migration.

Thus, we have been able to establish the liver as a site for the synthesis of α_1 -microglobulin. We have also demonstrated an immunosuppressive effect of the protein, a possible function for α_1 -microglobulin. It does not explain some of the intriguing properties of the protein, such as the binding in plasma to IgA and albumin, the charge-heterogeneity, and the brown-colored chromophore. More studies on α_1 -microglobulin will hopefully provide information sufficient to build a model for its biological actions, which includes an explanation of these properties.

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REFERENCES

- Balazs, T. and Roepke, R.R. (1966) Lysozymuria induced in rats by nephrotoxic agents. *Proc. Soc. Exp. Biol. Med.* 123, 380-385.
- Berggård, I. (1961) Studies on the plasma proteins in normal human urine. *Clin. Chim. Acta* 6, 413-429.
- Berggård, I. (1970) Proteinuria-mekanismer och karakteristiska urinproteiner. *Läkartidningen* 67, 5798-5803.
- Berggård, I. (1974) Isolation and characteristics of a rabbit β_2 -microglobulin: comparison with human β_2 -microglobulin. *Biochem. Biophys. Res. Commun.* 57, 1159-1165.
- Berggård, I. and Bearn, A.G. (1968) Isolation and properties of a low molecular weight β_2 -globulin occurring in human biological fluids. *J. Biol. Chem.* 243, 4095-4103.
- Berggård, I. and Edelman, G.M. (1963) Normal counterparts to Bence-Jones proteins: free L polypeptide chains of human γ -globulin. *Proc. Nat. Acad. Sci.* 49, 330-337.
- Berliner, R.W., Kennedy, T.J. and Hilton, J.G. (1950) Effect of maleic acid on renal function. *Proc. Soc. Exp. Biol. Med.* 75, 791-794.
- Bernier, I., Dautigny, A., Colombani, J. and Jollès, P. (1977) Human urine as a source of human leukocyte antigens. Purification and characterization of antigens from the first and second human leukocyte antigen locus: HLA-A9 and HLA-B12. *Biochim. Biophys. Acta* 490, 341-349.
- Bernier, I., Dautigny, A., Glatthaar, B.E., Lergier, W., Jollès, J., Gillesen, D. and Jollès, P. (1980) α_1 -microglobulin from normal and pathological urines. *Biochim. Biophys. Acta* 626, 188-196.
- Bernier, I., Dautigny, A., Jollès, J., Colombani, J. and Jollès, P. (1978) Molecular data on urinary glycoproteins with human leukocyte antigen (HLA) activity. *Biochim. Biophys. Acta* 533, 355-361.
- Dautigny, A., Bernier, I., Lethielleux, P., Colombani, J. and Jollès, P. (1979) Immunological and serological data concerning human histocompatibility antigens (HLA) and α_1 -microglobulin co-purified from urine. *Biomedicine* 31, 233-236.

- Ekström, B. and Berggård, I. (1977) Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J. Biol. Chem.* 252, 8048-8057.
- Ekström, B., Lundblad, A. and Svensson, S. (1981) Structural studies on the carbohydrate portion of human α_1 -microglobulin. *Eur. J. Biochem.* 114, 663-666.
- Ekström, B., Peterson, P.A. and Berggård, I. (1975) A urinary and plasma α_1 -glycoprotein of low molecular weight: Isolation and some properties. *Biochem. Biophys. Res. Commun.* 65, 1427-1433.
- Gahmberg, C.G. and Andersson L.C. (1978) Leukocyte surface origin of human α_1 -acid glycoprotein (orosomucoid) *J. Exp. Med.* 148, 507-521.
- Itoh, Y., Kin, K., Kasahara, T., Sakurabayashi, I., Kawai, K., Shiori-Nakano, K. and Takagi, K. (1979) Synthesis and secretion of α_1 -microglobulin by human lymphocytes. *Clin. exp. Immunol.* 37, 134-139.
- Koj, A. (1974) Acute phase reactants. In *Structure and function of plasma proteins* (Ed., Allison, A.C., Plenum Press, London) 1, 73-132.
- Lögdberg, L., Östergren, P.-O. and Berggård, I. (1979) Rat β_2 -microglobulin. Isolation, properties and relation to β_2 -microglobulins from other species. *Molecular Immunol.* 16, 577-587.
- Lopez, C., Grubb, A., Soriano, F. and Mendez, E. (1981) The complete amino acid sequence of human complex-forming glycoprotein heterogeneous in charge (protein HC). *Biochem. Biophys. Res. Commun.* 103, 191-925.
- Mendez, E., Grubb, A.O., Lopez, C., Frangione, B. and Franklin, E.C. (1982) Human complex-forming glycoprotein, heterogeneous in charge: The primary structure around the cysteine residues and characterization of a disulfide bridge. *Arch. Biochem. Biophys.* 213, 240-250.
- Pearlstein, E., Turesson, I., Tejler, L. and Grubb, A.O. (1977) Expression of protein HC on the plasma membrane of different human cell-types. *J. Immunol.* 119, 824-829.

- Peterson, P.A., and Berggård, I. (1971) Isolation and properties of a human retinol-transporting protein. *J. Biol. Chem.* 246, 25-33.
- Peterson, P.A., Evrin, P.-E. and Berggård, I. (1969) Differentiation of glomerular, tubular and normal proteinuria: determinations of urinary excretion of β_2 -microglobulin, albumin and total protein. *J. Clin. Invest.* 48, 1189-1198.
- Seon, B.K. and Pressman, D. (1978) Unique human glycoprotein, α_1 -microglycoprotein: Isolation from urine of a cancer patient and its characterization. *Biochemistry* 17, 2815-2821.
- Strober, W. and Waldmann, T.A. (1974) The role of the kidney in the metabolism of plasma proteins. *Nephron* 13, 35-66.
- Svensson, L. and Ravnskov, U. (1976) α_1 -microglobulin, a new low molecular weight plasma protein. *Clin. Chim. Acta* 73, 415-422.
- Takagi, K., Itoh, Y., Enomoto, H., Koyamaishi, Y., Maeda, K. and Kawai, T. (1980a) A comparative study of serum α_1 -microglobulin and β_2 -microglobulin levels in cancerous and other diseases. *Clin. Chim. Acta* 108, 277-283.
- Takagi, K., Kin, K., Itoh, Y., Enomoto, H. and Kawai, T. (1980b) Human α_1 -microglobulin levels in various body fluids. *J. Clin. Pathol.* 33, 786-791.
- Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T. and Shikata, T. (1979) Tissue distribution of human α_1 -microglobulin. *J. clin. Invest.* 63, 318-325.
- Takagi, T., Takagi, K. and Kawai, T. (1981) Complete amino acid sequence of human α_1 -microglobulin. *Biochem. Biophys. Res. Commun.* 98, 997-1001.
- Tejler, L. (1978) Protein HC. A low molecular weight plasma protein. *Ph.D. Thesis*. University of Lund, Lund, Sweden.
- Tejler, L., Eriksson, S., Grubb, A. and Åstedt, B. (1978) Production of protein HC by human fetal liver explants. *Biochim. Biophys. Acta* 542, 506-514.
- Tejler, L. and Grubb, A.O. (1976) A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine and cerebrospinal fluid. *Biochim. Biophys. Acta* 439, 82-94.

- Tejler, L., Grubb, A.O. and Turesson, I. (1976) Immunofluorescent demonstration of the presence of protein HC on the surface of human lymphocytes. *Acta Med. Scand.* 199, 425-427.
- Tomasi, T.B., Jr. (1977) Serum factors which suppress the immune response. In *Regulatory mechanisms in lymphocyte activation* (Ed., Lucas, D.O., Academic Press, New York)
- Touraine, J.L., Touraine, F., Revillard, J.P., Brochier, J. and Traeger, J. (1975) T-lymphocytes and serum inhibitors of cell-mediated immunity in renal insufficiency. *Nephron*, 14, 195-208.
- Vincent, C., Revillard, J.P., Bouic, P. and Brochier, J. (1982) Study of α_1 -microglobulin (HC Protein) with monoclonal antibodies. In *Protides of the biological fluids* (Ed., Peeters, H.) 29, 777-780.

Guinea-Pig α_1 Microglobulin

Isolation and Properties in Comparison with Human α_1 Microglobulin

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α_1 microglobulin has been isolated from urine of guinea-pigs with renal damage, induced by sodium chromate. Ultrafiltration, gel chromatography, zone electrophoresis and ion-exchange chromatography were used for the purification. The protein was shown to be homologous to human α_1 microglobulin, as the antiserum against this protein binds guinea-pig α_1 microglobulin. The molecular weight of the protein was 26000 when determined on sodium dodecyl sulfate polyacrylamide gel electrophoresis, 23000 as determined by gel chromatography in 6 M guanidine hydrochloride, and 24000 estimated from sedimentation and diffusion data. The amino acid content of guinea-pig α_1 microglobulin was similar to that of human α_1 microglobulin. The carbohydrate content was 16% and composed of sialic acid, glucosamine and neutral hexoses. The protein was markedly heterogeneous in charge. It was shown to consist of a number of subgroups with different isoelectric points, which showed extreme conservation between the guinea-pig and human α_1 microglobulin. The heterogeneity apparently does not reside in the carbohydrate moiety, since digestion with neuraminidase did not reduce the heterogeneity when tested on agarose gel electrophoresis. Only one band was observed when guinea-pig α_1 microglobulin was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions. The native protein has a tendency to form dimers. A brown substance is strongly bound to the protein and caused absorbance of light in the ultraviolet and visible regions. Fluorescence was observed in the high-frequency visible region. α_1 microglobulin was present in the serum of guinea-pigs at a mean concentration of 26.4 mg/ml as determined with radioimmunoassay. The antigenically active α_1 microglobulin was eluted as four components of different size when serum of guinea-pigs was chromatographed on a Sephadex G-200 column.

Human α_1 microglobulin is a glycoprotein present in plasma and urine [1]. It is heterogeneous in charge and binds a brown substance. The protein appears in free form as well as in complex with other plasma proteins. The molecular weight of human α_1 microglobulin is 26000. It has been isolated from patients with tubular proteinuria due to cadmium poisoning [1, 2]. Four other groups have studied human α_1 microglobulin [3–6]. It has been called protein HC [3] and α_1 microglycoprotein [5]. The biological significance of α_1 microglobulin is still unknown.

A purified homologue of α_1 microglobulin from a mammal other than man would greatly facilitate the delineation of the biological role of this protein. The demonstration itself of a homologue of α_1 microglobulin by biochemical and immunological criteria

in another mammal is of interest from an evolutionary point of view.

This report describes the isolation of guinea pig α_1 microglobulin from the urine of guinea-pigs with sodium-chromate-induced proteinuria. Such urine has been shown to be a good source for the preparation of the guinea-pig homologue of β_2 microglobulin [7]. Physicochemical properties of guinea-pig α_1 microglobulin demonstrate similarities with the human protein, and it is shown to cross-react immunologically with the human α_1 microglobulin.

MATERIALS AND METHODS

Urines and Sera

24-h urine specimens were collected from guinea-pigs treated with sodium chromate. The urine was

* Deceased June 1978.

Abbreviation. IgG, immunoglobulin G.

stored at -20°C with 0.1% NaN_3 and with pH adjusted to 6.5. Urine was collected for 5 to 7 days. The details of the sodium chromate treatment and the urine sample handling have been described earlier [7]. Sera from guinea-pigs were obtained by bleeding healthy animals via the carotid artery, and kept with 0.05% NaN_3 at -20°C until used.

Antisera

Antisera against purified guinea-pig α_1 microglobulin were prepared in two rabbits. 0.5 ml emulsion containing equal volumes of antigen (3 mg/ml in 0.02 M Tris-HCl + 0.15 M NaCl, pH 8.0, with 32 mg/ml benzylpenicillin, Kabi, Sweden) and Freund's complete adjuvans (Difco, U.S.A.) was injected intracutaneously in each rabbit. The rabbits were bled without boosting seven weeks after the injection. One of the antisera was absorbed by glutaraldehyde insolubilized serum from guinea-pigs. Monomeric α_1 microglobulin had been removed from the serum prior to the insolubilization procedure. The method for glutaraldehyde cross-linking and absorption has been described by Avrameas and Ternynck [8]. Antisera against human α_1 microglobulin were prepared as outlined earlier [2].

Other Materials

Ampholine polyacrylamide gel plates were obtained from LKB (Bromma, Sweden). DEAE-cellulose 23 SH was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.) and allowed to sediment in buffer to remove the finest particles. Pevikon C-870 was from Kema-Nord AB (Stockholm, Sweden). ^{125}I was a product of Amersham (Bucks, England), and iodo[^{14}C]acetamide was obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). Human serum albumin, human immunoglobulin G (IgG), chicken egg albumin, lactate dehydrogenase, human hemoglobin (type IV), and equine myoglobin were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Human α_1 microglobulin and human β_2 microglobulin were purified as described earlier [2, 9].

Labelling of Proteins with ^{125}I

Lactoperoxidase [10] was used to iodinate α_1 microglobulins for determination of molecular weight by gel chromatography in guanidine hydrochloride. All other radiolabellings were done with the chloramin-T procedure of Greenwood et al. [11].

Determination of Protein Concentrations

The radioimmunoassay described by Plesner et al. [12] was employed when determining α_1 microglobulin

concentrations smaller than 0.05 mg/ml. α_1 microglobulin at higher concentrations was determined with the single radial immunodiffusion technique outlined by Mancini et al. [13]. Total protein concentrations were measured with the modified method of Lowry et al. [14], with human IgG as standard, or by determining the absorbance at 280 nm.

Concentration of Protein Solutions

Concentration of urine, sera and other protein solutions were accomplished by ultrafiltration with 24/32-in (60/80-cm) Visking dialysis tubing (Union Carbide Corp., U.S.A.). The procedure has been described earlier [15].

Electrophoretic Methods

Preparative zone electrophoresis was performed in 0.1 M borate buffer, pH 8.9, using Pevikon C-870 as supporting medium. The processing was done as previously reported [16]. Agarose gel electrophoresis was carried out according to the method described by Johansson [17]. Disc polyacrylamide gel electrophoresis with sodium dodecyl sulfate was performed as outlined by Neville [18]. The running gel had a total acrylamide concentration of 12%, and the cross-linking was 3.3%. To determine the apparent molecular weight with this method, human serum albumin (68000), chicken egg albumin (43000), lactate dehydrogenase (36000), human α_1 microglobulin (31000), equine myoglobin (17200), and human β_2 microglobulin (11800) were used as markers with known molecular weight. The samples were sometimes treated with 2-mercaptoethanol as a reducing agent, prior to electrophoresis. Human hemoglobin was used as a marker with no intrachain disulfide bond. When separating radioactive samples, the gel rods were sliced into approximately 60 fractions which were analysed for radioactivity separately. Analytical isoelectric focusing on thin-layer gels was performed using Ampholine polyacrylamide gel plates (pH 3.5–9.5) as suggested by the instructions supplied.

Immunochemical Methods

Double radial immunodiffusion according to Ouchterlony, and immunoelectrophoresis were performed as described elsewhere [9]. Immunoprecipitation of radiolabelled protein was done as previously reported [19].

Determination of Molecular Weight by Gel Chromatography

Guinea-pig α_1 microglobulin was subjected to gel chromatography on a Sepharose CL-6B column, equili-

brated with 0.05 M sodium acetate buffer, pH 4.8, containing 6 M guanidine hydrochloride [20]. The details of the procedure have been described earlier [2]. When human and guinea-pig α_1 microglobulin were analyzed in the same experiment, one of the α_1 microglobulins was used in the ^{125}I -labelled form.

Estimation of Stokes' Radius

Stokes' radius was measured at 4°C by gel chromatography on a Sephadex G-200 column (100 $\times$ 0.9 cm) equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The experimental procedure has been described by Karlsson et al. [21]. Calculations were made according to Laurent and Killander [22]. The apparent diffusion coefficient was calculated using the Stokes-Einstein equation [23].

Ultracentrifugation

Sedimentation velocity experiments were performed in an MSE-75 Centriscan analytical ultracentrifuge, and the protein concentrations in the cells were determined with an ultraviolet scanner. 5-mm single-sector cells with quartz windows were used. Four different concentrations of α_1 microglobulin in 0.02 M Tris-HCl, pH 8.0, with 0.15 M NaCl and 0.02% NaN_3 were centrifuged at 53000 rev./min and 20°C. The distance from the center of rotation was measured every 20 min as position of half the maximal concentration. The partial specific volume used for calculation of the sedimentation coefficient ($s_{20, w}$) was derived from the amino acid and carbohydrate composition using the values of Gibbons [24], and Cohn and Edsall [25]. Svedberg's equation was employed to compute the molecular weight from the diffusion and sedimentation coefficients.

Amino Acid Analysis

Quantitative amino acid analyses, including determination of tryptophan and half-cystine, and analyses of free sulfhydryl groups were carried out as described for human α_1 microglobulin [2].

Determination of Carbohydrates

Neutral sugars were determined colorimetrically by the orcinol method of Vasseur [26], used as reported earlier [15]. Sialic acid content was measured by the Bial reaction according to Werner and Odin [27], or by the thiobarbituric acid assay of Warren [28]. Hexosamines were estimated in the amino acid analyzer after hydrolysis in 4 M HCl at 100°C for 5, 10, 16, and 22 h.

Optical Methods

Fluorescence emission and excitation spectra were recorded with an Aminco-Bowman spectrofluorimeter.

Guinea-pig α_1 microglobulin solutions of 0.4 mg/ml in 0.01 M phosphate buffer, pH 7.5, were used. The light absorption spectrum of guinea-pig α_1 microglobulin in 0.1 M phosphate buffer, pH 7.4, was determined in the ultraviolet and visible ranges in a Zeiss spectrophotometer, model PMQ II. The molar absorption coefficient was calculated after correction of the protein weight for ash and moisture. Moisture was determined by drying for 6 h at 105°C and 0.05 mm Hg over P_2O_5 , and ash by combustion in oxygen atmosphere at 800°C for 30 min.

Digestion with Neuraminidase

The digestion was carried out as described earlier [2].

RESULTS

Purification of Guinea-Pig α_1 Microglobulin

The isolation procedure is identical with that used when preparing human α_1 microglobulin with the exception that borate buffer instead of barbital buffer is used for guinea-pig α_1 microglobulin in the zone electrophoresis. As described below, α_1 microglobulin was shown to absorb light at 330 nm, and the absorbance at this wavelength was used to trace the protein specifically during the first preparations when no antiserum was available. These preparations resulted in the production of such antisera, which were used to trace the α_1 microglobulin in later purifications. The α_1 microglobulin could also be followed visually as a brown color during the isolation procedure. All operations were carried out at 4°C. Table 1 summarizes

Table 1. Purification of guinea-pig α_1 microglobulin

The urinary proteins were obtained by collecting 24-h specimens of urine from 84 male guinea-pigs treated with sodium chromate. Total protein was determined with the Folin technique modified by Lowry et al., by measuring the absorbance at 280 nm or by weighing the protein. α_1 microglobulin was measured by single radial immunodiffusion

Fraction	Total protein	α_1 microglobulin	Yield	Purity
	mg		%	
Concentrated urinary proteins	13571	531	100	4
First chromatography on Sephadex G-100	634	263	50	41
Zone electrophoresis	270	180	34	67
Second chromatography on Sephadex G-100	98	92	17	94
DEAE-cellulose chromatography	74	74	14	100

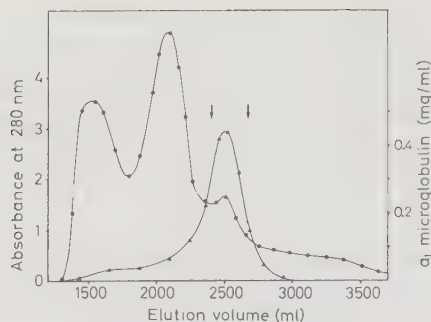


Fig. 1. Gel chromatography of concentrated urine from 20 male guinea-pigs, with sodium-chromate-induced proteinuria, on a Sephadex G-100 column (105×7 cm). The sample contained 3.2 g protein in 19 ml. 12-ml fractions were collected at a rate of 37 ml/h. α_1 microglobulin was measured by single radial immunodiffusion. Fractions to the next purification step were pooled as indicated by the arrows. (●) A_{280} ; (▲) concn of α_1 microglobulin

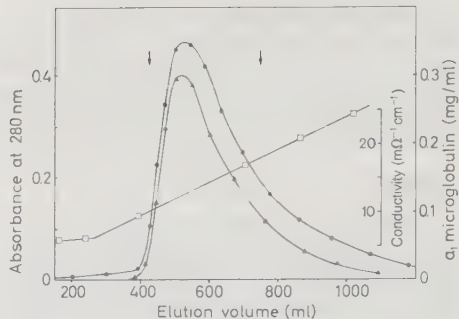


Fig. 2. Final DEAE-cellulose chromatography of α_1 microglobulin. 75 mg protein in 11 ml were applied to the column (4×11.5 cm) which was equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl. The column was eluted with linear gradient from 0.05 M to 0.30 M NaCl at a total volume of 1200 ml. 8-ml fractions were collected at a rate of 16 ml/h. Single radial immunodiffusion was used to trace α_1 microglobulin. Fractions were combined as shown by the arrows. (●) A_{280} ; (▲) α_1 microglobulin concn; (□) conductivity

the yield and purity from the different steps of a typical preparation.

First Gel Chromatography on Sephadex G-100. Urine from 21 guinea-pigs, treated with sodium chromate, was pooled and concentrated. After centrifugation at $15000 \times g$, the urine was chromatographed on a Sephadex G-100 column (113×8 or 105×7 cm). The column was equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The eluted fractions were analyzed for total protein by measuring the absorbance at 280 nm. Guinea-pig α_1 microglobulin was traced by single radial immunodiffusion, using the specific antiserum, and appeared as a peak eluted just after albumin. Fig. 1 shows the elution profile of this step and how the fractions were pooled.

Zone Electrophoresis in Borate Buffer. The α_1 microglobulin fractions from three or four gel chromatographies were pooled and concentrated before separating the proteins by preparative zone electrophoresis in 0.1 M borate buffer, pH 8.9. Some slowly migrating material was distinct from the majority of the proteins, which migrated as a broad peak toward the anode. Total protein was measured as absorbance at 280 nm, and fractions containing α_1 microglobulin were indicated by single radial immunodiffusion with antiserum to guinea-pig α_1 microglobulin. The protein was found in the slower part of the main peak, distributed in a wide zone. To avoid contaminants, many fractions containing α_1 microglobulin were not included in the pool subjected to the next isolation step.

Second Gel Chromatography on Sephadex G-100. The α_1 microglobulin fraction from the zone electrophoresis was concentrated and chromatographed on

Sephadex G-100. The column (105×7 or 104×5 cm) was equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The material was eluted as two distinct peaks, partially separated from each other and both included in the gel, when measured as absorbance at 280 nm. Guinea-pig α_1 microglobulin was present in both peaks. The ratio between α_1 microglobulin concentration, as measured by single radial immunodiffusion, and the absorbance at 280 nm, was approximately the same in both peaks, but lower in between these fractions. This indicates fairly pure α_1 microglobulin in the peaks and the presence of contaminants between the α_1 microglobulin fractions. The earlier peak was eluted at a position that would be expected from a molecule with twice the size of the substance in the slower peak. This pattern with two separate peaks containing α_1 microglobulin reappeared when rechromatographing material from either peak, indicating a spontaneous formation of dimeric guinea pig α_1 microglobulin, which is in equilibrium with the monomeric form. Both α_1 microglobulin fractions were pooled.

Chromatography on DEAE-Cellulose. The slow α_1 microglobulin peak was concentrated and dialyzed against 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl. The sample was applied to a DEAE-cellulose column, equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride between 0.05 M and 0.30 M. The elution of the column was followed by measuring the absorbance at 280 nm and by single radial immunodiffusion of guinea-pig α_1 microglobulin. Fig. 2 shows the result. α_1 microglobulin appears to be heterogeneous in charge as judged by the asymmetric elution pattern. The frac-

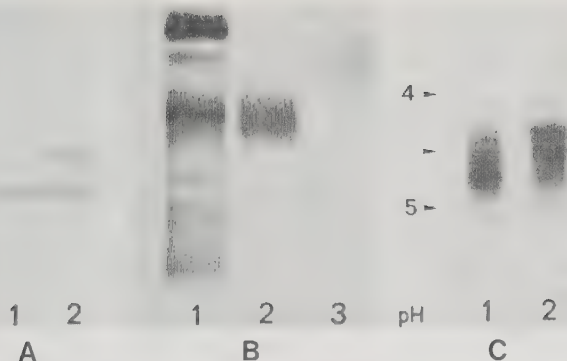


Fig. 3. Electrophoresis and isoelectric focusing of α_1 microglobulin. (A) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of guinea-pig α_1 microglobulin alone (1) and mixed with human α_1 microglobulin (2). Total acrylamide concentration was 12% and the cross-linking 3.3%. The anode is at the bottom. (B) Agarose gel electrophoresis of serum from a guinea-pig (1), guinea-pig α_1 microglobulin treated with neuraminidase (2), and untreated α_1 microglobulin (3). The anode is at the top. (C) Isoelectric focusing on thin-layer polyacrylamide gel of human α_1 microglobulin (1) and guinea-pig α_1 microglobulin (2). A pH gradient was established between 3.5 and 9.5. The anode is at the top.

tions were pooled as in Fig. 2, and dialyzed against distilled and deionized water, and lyophilized.

Size Homogeneity and Charge Heterogeneity of Guinea-Pig α_1 Microglobulin

The isolated protein migrated as a homogeneous band when analyzed on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The size homogeneity is evident from Fig. 3A, showing guinea-pig α_1 microglobulin compared to the human α_1 microglobulin. Gel chromatography of the protein in the presence of 6 M guanidine hydrochloride gave one symmetrical peak. To investigate the size homogeneity of α_1 microglobulin in the urine of guinea-pigs, fractions eluted earlier than the main α_1 microglobulin fractions in the first gel chromatography step were radio-iodinated with ^{125}I and submitted to immunoprecipitation with absorbed antiserum to guinea-pig α_1 microglobulin and goat anti-rabbit IgG. When analyzing the immunoprecipitate from fractions of two different elution volumes on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, only one component of 26000 was seen in all experiments. On agarose gel electrophoresis, α_1 microglobulin showed the pronounced heterogeneity of charge, which was also seen during the purification of the protein. Removing the sialic acid with neuraminidase did not affect the charge heterogeneity, although α_1 microglobulin devoid of sialic acid migrated considerably slower (α_2 region) than the untreated protein (Fig. 3B). The charge heterogeneity of guinea-pig and human α_1 microglobulin is also illustrated in Fig. 3C, showing isoelectric focusing of the

proteins. Three double bands and two additional cathodal bands were distributed between pH 4.3 and 4.8. This characteristic pattern could be reproduced with human α_1 microglobulin from three different individuals, and with guinea-pig α_1 microglobulin from six different preparations.

Physical and Chemical Properties of Guinea-Pig α_1 Microglobulin

Shape and Size. Five determinations of Stokes' radius of two preparations of guinea-pig α_1 microglobulin gave an average value of 2.52 nm (S.E. = 0.01 nm). The sedimentation coefficient was determined to 2.46 S (S.E. = 0.02 S) for two preparations using four different concentrations (0.04–0.25 mg/ml) of the protein. The molecular weight was 25800 (S.E. = 200) determined with polyacrylamide gel electrophoresis in sodium dodecyl sulfate in ten experiments for two preparations of guinea-pig α_1 microglobulin. Four chromatographies on a Sepharose CL-6B column in 6 M guanidine hydrochloride gave a mean molecular weight of 23000 (S.E. = 100). A value of 24000 was obtained when calculating the molecular weight from the sedimentation and diffusion coefficients. The presence of at least one disulfide loop in guinea-pig and human α_1 microglobulin is indicated by the fact that the proteins migrated slower in the presence of 2-mercaptoethanol or dithioerythritol than when no reducing agent was present when submitting to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Table 2 compares some physical

Table 2. *Physical and chemical properties of guinea-pig α_1 microglobulin compared to human α_1 microglobulin*

Experiments to obtain the figures were carried out as described in the text. All data for the human protein are taken from [2] except the isoelectric points

Parameter	Guinea-pig	Man
Sedimentation coefficient, $s_{20,w}$ (S)	2.46	2.35
Stokes' molecular radius, r_s (nm)	2.52	2.85
Partial specific volume, v_2 (ml/g)	0.709	0.704
Apparent diffusion coefficient, $D_{20,w}$ ($\mu\text{m}^2 \text{s}^{-1}$)	85	75
Frictional ratio, f/f_0	1.34	1.45
Molecular weight		
By sedimentation and diffusion coefficient	24000	26100
By gel chromatography in 6 M guanidine hydrochloride after reduction and alkylation	23000	24800
By dodecyl sulfate/polyacrylamide gel electrophoresis	25800	31000
Absorption coefficient at 280 nm, ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	5.14×10^4	4.72×10^4
Carbohydrate content (%)		
Hexose	7.7	6.4
Glucosamine	3.8	5.1
Sialic acid	4.2	5.5
pI	4.3–4.8	4.3–4.8

Table 3. *Amino acid composition of guinea-pig α_1 microglobulin compared to human α_1 microglobulin*

The figures of the guinea-pig protein ($\pm$ S.E.) are calculated on the basis of a molecular weight of 23500. Threonine, serine and tyrosine were determined after extrapolation to 0 h hydrolysis, cysteine was determined in three experiments as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride, valine was determined from 72 h hydrolysis only, tryptophan was determined spectrophotometrically in seven different experiments. All other figures for guinea-pig α_1 microglobulin are averages of values from one 24-h and one 72-h hydrolysis in 19 different experiments. The human data are taken from [2]

Amino acid	Guinea-pig	Man
	residues/mol	
Aspartic acid/asparagine	15.8 ± 0.04	14.3
Threonine	15.6 ± 0.03	16.9
Serine	12.4 ± 0.03	10.3
Glutamic acid/glutamine	22.1 ± 0.05	21.7
Proline	10.8 ± 0.04	13.5
Glycine	13.5 ± 0.05	14.0
Alanine	9.7 ± 0.01	9.3
Cysteine	3.2 ± 0.13	2.3
Valine	8.4 ± 0.03	10.9
Methionine	4.7 ± 0.01	4.5
Isoleucine	7.0 ± 0.03	12.4
Leucine	13.3 ± 0.03	11.0
Tyrosine	6.9 ± 0.04	8.3
Phenylalanine	5.0 ± 0.03	6.2
Lysine	9.3 ± 0.02	10.6
Histidine	3.8 ± 0.02	3.6
Arginine	10.6 ± 0.03	9.7
Tryptophan	3.3 ± 0.03	3.7
Total	175.4	183.2

and chemical properties of guinea-pig α_1 microglobulin with those of the human protein.

Amino Acid and Carbohydrate Content. The amino acid figures presented in Table 3 are averages of 19 analyses of four different preparations. Values for each analysis were derived from one 24-h hydrolysis and one 72-h hydrolysis and were calculated on the basis that the weight of all amino acids should amount to 23500 minus the weight of the carbohydrates. When cysteine and half-cystine were determined as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride the value was estimated at 3.2. 4.4 residues were indicated when determining cysteic acid after performic acid oxidation. When alkylating the non-reduced protein in the presence of 6 M guanidine hydrochloride no free sulfhydryl group could be detected. Carbohydrates made up 16% of the total weight of guinea-pig α_1 microglobulin. Sialic acid contributed with 4.4% when assayed with the Bial reaction, and with 3.9% when the thiobarbituric acid assay was used. Glucosamine amounted to 4.3% of the weight of α_1 microglobulin. This figure was obtained by hydrolysis at 100°C with 4 M HCl for 10 h, which always yielded higher values than 5, 16 or 22 h of hydrolysis. Only traces of galactosamine could be detected with this method. The content of neutral sugars was 7.7%. The values for the carbohydrate content are given in Table 2.

Optical Properties. The molar absorption coefficient at 280 nm of guinea-pig α_1 microglobulin was determined to $5.14 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$. The absorption spectrum showed a maximum at 278 nm, and a plateau starting at approximately 300 nm decreased slowly. The absorbance extended throughout the visible region, but did not show any maxima. The spectrum is illustrated in Fig. 4A.

The fluorescence excitation and emission spectra of α_1 microglobulin are shown in Fig. 4B. The excitation spectrum has a maximum at 290 nm, and another, more diffuse maximum at 380 nm, when measuring the emission at 475 nm. The emission spectrum reveals that the emission of light, when exciting the protein at 380 nm, is most intense somewhere between 475 and 500 nm.

Presence of Guinea-Pig α_1 Microglobulin in Urine and Serum

Antiserum to guinea-pig α_1 microglobulin could precipitate material in urine and serum of guinea-pigs. The precipitation reactions were of immunological identity with purified α_1 microglobulin when analyzed with the Ouchterlony immunodiffusion technique. The concentrations of α_1 microglobulin were determined in urine and serum from healthy guinea-pigs and in urine from guinea-pigs treated with sodium chromate. The serum concentration was 26.4 mg/l (S.E.

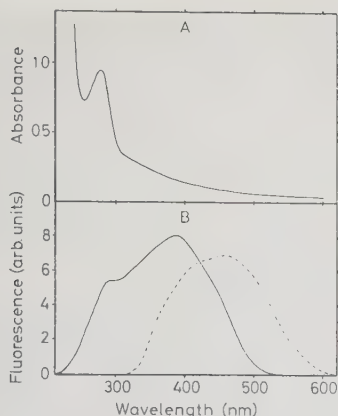


Fig. 4. (A) Absorption spectrum of guinea-pig α_1 microglobulin between 240 and 600 nm. (B) Fluorescence spectra of guinea-pig α_1 microglobulin (0.4 mg/ml) in 0.01 M phosphate buffer, pH 7.5. (A) A solution of 0.44 mg/ml protein in 0.1 M phosphate buffer, pH 7.4, was used. (B) The excitation spectrum (—) was obtained by recording the light emission at 475 nm, and the emission spectrum (---) by exciting at 380 nm

= 1.0 mg/l), determined with radioimmunoassay on seven guinea-pigs. The content of α_1 microglobulin in the normal urine was 1.4 mg/24-h volume (S.E. = 0.2 mg/24-h vol.) and 4.6 mg/24-h volume (S.E. = 0.3 mg/24-h vol.) in the urine collected the first 6 days after the injection of sodium chromate in three groups of 20 guinea-pigs. The urine was analyzed with radioimmunoassay and single radial immunodiffusion. When chromatographing serum on a Sephadex G-200 column, the α_1 microglobulin activity, as measured by radioimmunoassay, is eluted as four components differing in apparent size (Fig. 5). The smallest of these components is eluted at the position of urinary α_1 microglobulin. The three peaks eluted earlier are less distinct, especially the one eluted with the void volume.

Immunological Cross-Reaction between Human and Guinea-Pig α_1 Microglobulin

Antiserum to human α_1 microglobulin could bind 125 I-labelled guinea-pig α_1 microglobulin and antiserum to guinea-pig α_1 microglobulin could bind 125 I-labelled human α_1 microglobulin when separating the antibody-antigen complexes from the unbound antigens with 10% polyethyleneglycol. Both these bindings could be totally inhibited by purified guinea-pig α_1 microglobulin or by human α_1 microglobulin. Antiserum to human α_1 microglobulin could not precipitate guinea-pig α_1 microglobulin, nor could antiserum to guinea-pig α_1 microglobulin precipitate human α_1

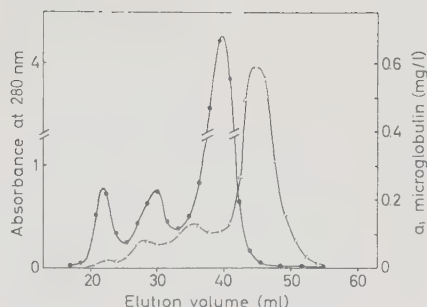


Fig. 5. Gel chromatography on Sephadex G-200 of serum from a healthy guinea-pig. The column (93 $\times$ 1 cm) was equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. The sample, 1.1 ml, was eluted at a flow rate of 1.9 ml/h. Fractions of 0.8 ml were collected and α_1 microglobulin concentrations were determined with radioimmunoassay. (●) A_{280} ; (Δ) α_1 microglobulin concn

microglobulin when tested with Ouchterlony immunodiffusion.

DISCUSSION

The guinea-pig α_1 microglobulin described in this work is homologous to human α_1 -microglobulin for a number of criteria. The homology is demonstrated by the similarity between the two proteins with respect to the amino acid and carbohydrate composition, the dimerization and the isoelectric focusing pattern, the presence of a brown-colored material, and some physicochemical constants describing the size and shape of the proteins. The immunological cross-reaction between human and guinea-pig α_1 microglobulin shows that they are homologous with at least one antigenic determinant in common.

Sodium chromate has successfully been used to induce proteinuria in guinea-pigs, and β_2 microglobulin has been purified from such urine [7]. In this study, α_1 microglobulin was isolated from urine of guinea-pigs treated with sodium chromate. The protein could be purified using essentially the same techniques as for human α_1 microglobulin [1, 2].

The molecular weight of guinea-pig α_1 microglobulin (23 500) is smaller than the molecular weight of human α_1 microglobulin (26 000) [2]. This difference may partly be due to the fact that the guinea-pig homologue contains a smaller amount of carbohydrates, and partly to a difference in the length of the polypeptide. One may argue that the guinea-pig

α_1 microglobulin described in this work could be a degradation product of a urinary component with a molecular weight closer to that of human α_1 microglobulin. No such larger component could be detected when subjecting early fractions of gel-chromatographed urine to immunoprecipitation with antiserum to guinea-pig α_1 microglobulin followed by analysis on sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

The charge heterogeneity observed in the isolation procedure seems to be caused by a limited number of subpopulations of the protein with different isoelectric points, as judged by thin-layer isoelectric focusing. The patterns of the two homologues are strikingly similar and reproducible within and between the species. No difference could be seen between human α_1 microglobulins prepared from different individuals. This extreme conservation of the isoelectric points suggests that the charge of the protein may be highly important to its function.

Since the charge heterogeneity may, at least partly, reside in the primary structure of the polypeptide chain, the values obtained by amino acid analysis might represent a mixture of subpopulations of α_1 microglobulin with different amino acid compositions. For this reason the values of the amino acids have not been extrapolated to the nearest integer. The calculations were not, for the same reason, made by assuming a certain amount of a particular amino acid, or by adjusting the figures to come as close as possible to an integer for all amino acids. The values are based on the assumption that the total weight of the amino acids amounts to 84% of the molecular weight of guinea-pig α_1 microglobulin (23 500). This also assumes that the weight of the chromophore material is negligible. The amino acid composition is very similar to that of human α_1 microglobulin. One exception to this isoleucine, of which the guinea-pig homologue contains 7.0 residues and the human protein 12.4.

Guinea-pig α_1 microglobulin contains 3.2 half-cystine residues per molecule when measured as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride followed by dialysis, and 4.4 residues when estimated as cysteic acid after performic acid oxidation. The values for human α_1 microglobulin were 2.3 and 3.4 respectively [2]. It is possible that the protein subgroups contain varying amounts of half-cystine, and that the dissimilarities between the two homologues reflect different combinations of these subgroups. As for human α_1 microglobulin, the lower value obtained after reduction and dialysis of guinea-pig α_1 microglobulin indicates the presence of a free, dialyzable cysteine molecule involved in an S-S bond with a half-cystine residue. Both human and guinea-pig α_1 microglobulin migrated faster without any reducing agent than in the presence of 2-mercaptoethanol or dithioerythritol, when sub-

jected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Hemoglobin, as an internal marker without any intra-chain S-S bond, showed the same mobility with or without any 2-mercaptoethanol or dithioerythritol. This suggests the presence of a disulfide bond in both homologues. Previous data indicated the presence, if at all, of only a small disulfide loop [2]. Seon and Pressman propose two S-S bonds, since they found 3.7 half-cystine residues after reduction, alkylation and dialysis in 7 M guanidine hydrochloride [5]. However, they present no other results which support that postulate.

A brown-colored material, similar to that bound to human α_1 microglobulin [2], is also linked to the guinea-pig homologue. The substance absorbs light in the ultraviolet and visible regions in an undefined manner which indicates the presence of a heterogeneous material. The absorbed light with wavelengths between 300 and 400 nm was emitted as fluorescence scattered between 400 and 500 nm, in a manner supporting the heterogeneity of the material with optical activity.

The elution behaviour of material reacting with antiserum to human α_1 microglobulin, when chromatographing human serum on a Sephadex G-200 column [1], could be reproduced with guinea-pig serum. The second largest of the four components (Fig. 5) may correspond to the α_1 -microglobulin-IgA complex of human serum, reported by Tejler and Grubb [3]. This component of guinea-pig serum was not as dominant as in human plasma. The discrepancy may be caused by different affinities of the antisera to the α_1 microglobulin components present in the sera. The concentration of IgA in guinea-pig serum is about thirty times lower than in human serum, and the ratio monomeric to dimeric IgA is appreciably higher in the guinea-pig than in man [29]. These differences may also yield different elution profiles of α_1 microglobulin when gel chromatographing serum from the two species.

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REFERENCES

1. Ekström, B., Peterson, P. A. & Berggård, I. (1975) *Biochem. Biophys. Res. Commun.* **65**, 1427–1433.
2. Ekström, B. & Berggård, I. (1977) *J. Biol. Chem.* **252**, 8048–8057.
3. Tejler, L. & Grubb, A. O. (1976) *Biochim. Biophys. Acta*, **439**, 82–94.
4. Svensson, L. & Ravnskov, U. (1976) *Clin. Chim. Acta*, **73**, 415–422.
5. Seon, B. K. & Pressman, D. (1978) *Biochemistry*, **17**, 2815–2821.

6. Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T. & Shikata, T. (1979) *J. Clin. Invest.* **63**, 318–325.
7. Cigén, R., Ziffer, J. A., Berggård, B., Cunningham, B. A. & Berggård, I. (1978) *Biochemistry*, **17**, 947–955.
8. Avrameas, S. & Ternynck, T. (1969) *Immunochemistry*, **6**, 53–66.
9. Berggård, I. & Bearn, A. G. (1968) *J. Biol. Chem.* **243**, 4095–4103.
10. Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–305.
11. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123.
12. Plesner, T., Nørregaard-Pedersen, B. & Boenisch, T. (1975) *Scand. J. Clin. Lab. Invest.* **35**, 729–735.
13. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry*, **2**, 235–254.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
15. Berggård, I. (1961) *Ark. Kemi*, **18**, 291–313.
16. Peterson, P. A. & Berggård, I. (1971) *J. Biol. Chem.* **246**, 25–33.
17. Johansson, B. G. (1972) *Scand. J. Clin. Lab. Invest.* **29**, Suppl. 124, 7–19.
18. Neville, D. M., Jr (1971) *J. Biol. Chem.* **246**, 6328–6334.
19. Åkerström, B., Nilsson, K., Berggård, B. & Berggård, I. (1979) *J. Immunol.* **122**, 2516–2520.
20. Mann, K. G. & Fish, W. W. (1972) *Methods Enzymol.* **26**, 28–42.
21. Karlsson, F. A., Peterson, P. A. & Berggård, I. (1972) *J. Biol. Chem.* **247**, 1065–1073.
22. Laurent, T. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330.
23. Gosting, L. J. (1956) *Adv. Protein Chem.* **11**, 449.
24. Gibbison, R. A. (1972) in *Glycoproteins* (Gottschalk, A., ed.) p. 78, Elsevier, Amsterdam.
25. Cohn, E. J. & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides* (Cohn, E. J. & Edsall, J. T., eds) pp. 370–381, Reinhold, New York.
26. Vasseur, E. (1948) *Acta Chem. Scand.* **2**, 693–701.
27. Werner, I. & Odin, L. (1952) *Acta Soc. Med. Upsal.* **57**, 230–241.
28. Warren, L. (1959) *J. Biol. Chem.* **243**, 1971–1975.
29. Vaerman, J. P. & Heremans, J. F. (1972) *J. Immunol.* **108**, 637–648.

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TISSUE DISTRIBUTION OF GUINEA PIG α_1 -MICROGLOBULIN

by

Bo Åkerström

ABSTRACT

The levels of α_1 -microglobulin in various guinea pig organs were determined after homogenization followed by solubilization with sodium deoxycholate or sonication. Higher amounts were found in the kidney and liver than in the spleen, placenta, adrenal glands, heart, brain, lungs, abdominal muscle and testes. No α_1 -microglobulin could be detected in solubilized lymphocytes or erythrocytes. Subcellular fractions from the kidney, liver, placenta and muscle were prepared by differential centrifugation. Liver α_1 -microglobulin was found in the microsomal fraction (25%) and in the 105,000 x G supernatant (67.5%). Kidney, placenta and muscle α_1 -microglobulin was found in the 105,000 x G supernatants (89%, 88% and 100%, respectively). α_1 -microglobulin was accumulated in the medium of liver cells incubated for four hours. The accumulation could be inhibited by 1.5 mM sodium azide.

INTRODUCTION

α_1 -microglobulin was originally isolated from human urine (Ekström *et al.*, 1975, Ekström and Berggård, 1977). It is a low molecular weight plasma protein (molecular weight: 26,000) and contains approximately 20% carbohydrate. It is present in plasma in free form, as well as in high molecular weight complexes. The protein has been ascribed immunoregulatory properties: it inhibits *in vivo* antigen-induced lymphocyte transformation and stimulates the migration of leukocytes (Lögdberg and Åkerström, 1981). α_1 -microglobulin has also been purified and characterized by Tejler and Grubb, who called it protein HC (Tejler and Grubb, 1976). The protein was detected, with immunofluorescence, on the surface of lymphocytes, erythrocytes and fibroblasts (Pearlstein *et al.*, 1977). Another group reported staining of the surface of T- and B-lymphocytes with anti- α_1 -microglobulin (Takagi *et al.*, 1979), and demonstrated the presence of α_1 -microglobulin in culture-media of lymphocytes (Takagi *et al.*, 1979, Itoh *et al.*, 1979). We were able to show that the immunofluorescence on these cells by anti- α_1 -microglobulin-sera were probably due to contaminations in the preparations of α_1 -microglobulin, and we did not detect any release of the protein from cultured lymphocytes (Åkerström *et al.*, 1979). Thus, the origin and localization of α_1 -microglobulin are still unclear. However, the synthesis of α_1 -microglobulin from human fetal liver explants has been demonstrated (Tejler *et al.*, 1978).

Guinea pig α_1 -microglobulin has been purified and characterized (Åkerström and Berggård, 1979), and the investigation in this paper was designed to explore the role of various guinea pig tissues in the metabolism of α_1 -microglobulin. The tissue distribution, the subcellular localization and the release of α_1 -microglobulin from liver cells were studied.

MATERIALS AND METHODS

Proteins and antisera

Guinea pig α_1 -microglobulin was prepared from the urine of guinea pigs with sodium chromate-induced renal damage (Åkerström and Berggård, 1979). Guinea pig albumin was prepared by separating 20 ml guinea pig serum by preparative zone electrophoresis in 0.1 M barbital buffer, pH 8.6, using Pevikon C-870 (Kema-Nord AB, Stockholm, Sweden) as supporting medium. The procedure has been detailed by Peterson and Berggård (1971). Albumin-rich fractions were subjected to gel-chromatography on a Sephadex G-200 column (140 x 5 cm), eluted with 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The albumin, thus purified, was dialyzed against distilled and deionized water, and lyophilized. Antisera against guinea pig α_1 -microglobulin and albumin were prepared in rabbits as described (Åkerström and Berggård, 1979), and the anti- α_1 -microglobulin was absorbed with glutaraldehyde-insolubilized guinea pig serum (Avrameas and Ternynck, 1969).

Guinea pig tissues

Outbred guinea pigs were anesthetized with 45 mg mebumal (ACO, Sweden)/kg bodyweight, injected intraperitoneally. 1 ml 0.1 M sodium-citrate + 0.05 M NaCl was injected into the left renal vein. After 1 min., thorax was opened and a perfusion of the animal was carried out by injecting 0.5 l/kg bodyweight of the sodium citrate solution into the left ventricle at a flow-rate of 0.5-0.7 l/hour. The circulatory system was opened at the left renal vein to permit outflow. Specimens from the organs listed in Table I were rinsed with cold 0.15 M NaCl and frozen immediately at -80°C . Placentae were taken from guinea pigs in their 60th - 63rd day of gestation. Tissues from eight animals were collected. Peripheral blood lymphocytes were prepared as described by Böyum (1968). Erythrocytes were purified by passing the sediment of defibrinated blood (Åkerström *et al.*,

1979) through a cotton wool column and three successive centrifugations on Lymphoprep, density 1.077 (Nyegaard & Co., Norway), followed by washing with phosphate-buffered saline, pH 7.4.

Solubilization of tissues and blood cells

The tissues were thawed, and approximately 1 g (exact weight was noted) of each organ was homogenized in 3 ml ice-cold 50 mM Tris-HCl, pH 8.5, containing 0.25 M sucrose, 1 mM EDTA, and 1 mM diisopropyl-phosphofluoridate (DFP). The homogenization was carried out in a Potter-Elvehjem homogenizer, using 10 strokes with a teflon pestle. Another 2 ml of the ice-cold buffer was added to the homogenate, which was then divided into three aliquots. One of these was subjected to sonication 2 x 10 sec., and to another was added deoxycholate to 0.5%. After 10 min., all three aliquots were centrifuged at 15,000 x G for 45 min. at 4°C. The concentrations of α_1 -microglobulin and albumin were determined in the supernatants.

The peripheral blood lymphocytes and erythrocytes were solubilized by sonication followed by treatment with acid, deoxycholate, or NaSCN, as described by Björck *et al.*, (1979). After centrifugation at 105,000 x G for 75 min. at 4°C, the supernatants were assayed for α_1 -microglobulin.

Subcellular fractionation of tissues

Approximately 3 g (exact weight was noted) each of kidney, liver, placenta and muscle were homogenized in 5 ml ice-cold 50 mM Tris-HCl, pH 8.5, + 0.25 M sucrose, 1 mM EDTA and DFP, with a Potter-Elvehjem loose-fitting teflon pestle (5 strokes). Another 5 ml of the buffer was added, and the suspension was centrifuged at 1,000 x G for 10 min. at 4°C. The precipitates were washed once, and the supernatants were transferred to a new set of tubes, and centrifuged at 10,000 x G for 40 min. at 4°C. These precipitates were saved, and the supernatants were subjected to a final centrifugation at 105,000 x G for 60 min. at 4°C. The three sets of precipitates thus formed were suspended in 2 ml of the ice-cold Tris-sucrose buffer, and nonidet P-40 was

added to 1% to these suspensions and to 2 ml of the 105,000 x G supernatants. After 10 min. at 4°C, all fractions were centrifuged at 105,000 x G for 60 min. at 4°C, and the concentrations of α_1 -microglobulin and albumin were determined in the supernatants.

Liver cell preparations and incubations

A guinea pig liver cell suspension was prepared principally following the two-step perfusion-technique for rat-liver cells outlined by Seglen (1973), using the apparatus described by Nilsson *et al.* (1973). Briefly, the liver was first perfused *in situ* through v. porta (outflow through the lower v. cava) with an oxygenated, calcium-free Hanks' solution (Hanks and Wallace, 1949), substituting the bicarbonate and phosphate with 40 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (Hepes), at 37°C and a flow-rate of 20 - 30 ml/min. After 10 min., the liver was transferred to a Buchner funnel, collagenase (Sigma, type I) and CaCl_2 were added to 0.05% and 1.3 mM respectively to the perfusion medium, which was now recirculated. After 20 - 30 min., the liver was cut into pieces, filtered through a nylon filter (100 micromesh), centrifuged twice at 40 x G for 2 min. and finally filtered again through a nylon filter. The cells were counted in a Bürker chamber and the viability was tested with 0.05% trypan blue (more than 80% viability). The liver cells were incubated at 37°C in the modified Hanks' solution, now buffered with 20 mM Hepes (10^6 cells/ml) with or without the addition of 1.5 mM NaN_3 . The cells were maintained suspended by rotatory shaking. After 10 min. or 4 hours, the suspensions were centrifuged at 40 x G for 5 min. The supernatants were decanted and centrifuged at 1,000 x G for 20 min., and then assayed for α_1 -microglobulin.

Determination of protein concentrations

α_1 -microglobulin concentrations were determined with the radioimmunoassay described by Plesner *et al.* (1975), using absorbed, whole antiserum diluted to a binding capacity of 50% of the labeled α_1 -microglobulin. Solubilized whole tissues were

diluted with 50 mM Tris-HCl, pH 8.5, + 0.25 M sucrose and 0.1% bovine serum albumin, and solubilized subcellular fractions were diluted with the same buffer also containing 1% nonidet P-40, before tested in the radioimmunoassay. Standard α_1 -microglobulin was diluted in the same buffer as the sample subjected to the radioimmunoassay. α_1 -microglobulin was radiolabeled with ^{125}I (Amersham, England) by the chloramin-T-method (Greenwood and Hunter, 1963) or with lactoperoxidase (Marchalonais, 1969) using a radioiodination kit (NEZ 151, New England Nuclear, Boston, USA) as suggested by the instruction supplied. Albumin concentrations were estimated by single radial immunodiffusion (Mancini *et al.*, 1965).

Other methods

Concentration of protein solutions were accomplished by ultrafiltration through 24/32 inch Visking dialysis tubings (Union Carbide Corp., USA) (Berggård, 1961).

RESULTS

Radioimmunoassays

The inhibitory capacity of the solubilized tissues and subcellular fractions on the binding of radiolabeled α_1 -microglobulin to antiserum was tested by serial dilutions of the 105,000 x G supernatants. Figure 1 shows the inhibition curves of solubilized liver and kidney, compared to urinary α_1 -microglobulin and guinea pig serum. Similar patterns were obtained whether the tissues were treated with homogenization only, homogenization and deoxycholate, or homogenization and sonication. The maximal inhibition of ^{125}I - α_1 -microglobulin-binding was 90-95% for the kidney homogenates and 70-80% for the liver homogenates. At low concentrations, the inhibition curves of the solubilized tissues and subcellular fractions seemed to parallel those of standard α_1 -microglobulin. Inhibitions greater than 50% were disregarded as measurements of α_1 -microglobulin. The inhibition profile of urinary α_1 -microglobulin was not

affected by homogenization, sonication or treatment with deoxycholate, nonidet P-40, NaSCN or acid.

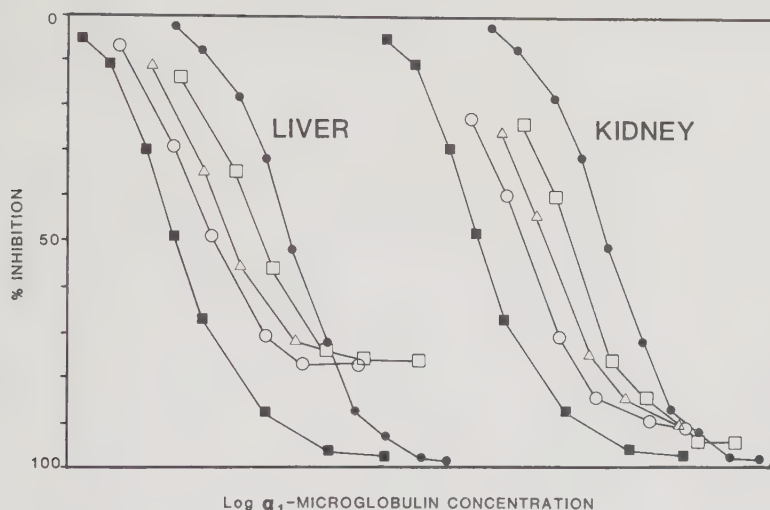


Fig. 1. Inhibition of the binding ^{125}I -labeled urinary guinea pig α_1 -microglobulin to anti-guinea pig α_1 -microglobulin by non-labeled urinary α_1 -microglobulin ($\bullet$ — $\bullet$), guinea pig serum ($\blacksquare$ — $\blacksquare$), and α_1 -microglobulin in guinea pig liver and kidney, solubilized by homogenization ($\square$ — $\square$), homogenization followed by treatment with 0.5% deoxycholate (Δ — Δ), or homogenization followed by sonication 2×10 seconds ($\circ$ — $\circ$). The dilution series of the five solutions are not correlated to each other on the x-axis.

α_1 -microglobulin concentration in tissues

Table I shows the yield of α_1 -microglobulin ($\mu\text{g/g}$ of tissue) when solubilizing various tissues by homogenization only (I), homogenization followed by treatment with 0.5% deoxycholate (II), or followed by sonication for 2×10 sec. (III). The highest values for α_1 -microglobulin were found in the kidney. In the liver, lower amounts were detected although these were always higher than the levels found in other tissues. Solubilized lymphocytes and erythrocytes failed to display any α_1 -microglobulin. The detection limit was approximately $\ln\text{g}/10^6$ cells for the lymphocytes and $1 \text{ ng}/10^9$ cells for the red

blood cells. Significant amounts of the α_1 -microglobulin detected in the tissues may originate from serum, due to incomplete perfusion of the organs.

Table I
Tissue distribution of α_1 -microglobulin and albumin

Tissue	α_1 -microglobulin [•] µg/g tissue [†]			Albumin [•] mg/g tissue [†]			α_1 -microglobulin/Albumin [‡] $\times 10^3$		
	I	II	III	I	II	III	I	II	III
Kidney	35.0	37.2	33.4	3.1	2.7	3.0	14.6	17.9	14.8
Liver	6.5	7.1	6.2	1.2	1.3	1.3	5.7	6.1	5.5
Spleen	3.9	3.1	3.6	1.7	1.8	1.7	2.3	1.7	2.2
Placenta	4.9	4.8	5.5	3.0	2.9	2.9	1.7	1.7	1.9
Adrenal glands	1.5	1.2	1.3	1.1	1.1	1.2	1.6	1.3	1.3
Heart	3.3	2.7	2.9	2.8	2.8	2.9	1.3	1.1	1.1
Brain	0.062	0.030	0.068	0.045	0.034	0.053	1.4	0.9	1.3
Lungs	4.3	3.8	4.0	3.7	3.9	3.6	1.2	1.1	1.2
Muscle (abdominal)	1.3	1.1	1.2	1.5	1.3	1.4	0.9	0.8	0.9
Testes	1.8	1.8	2.0	2.3	2.5	2.6	0.8	0.7	0.8

I: obtained by homogenization

II: obtained by homogenization and treatment with 0.5% deoxycholate

III: obtained by homogenization and sonication 2×10 seconds

• Values are means of eight samples

† Wet weight

‡ Values are means of the ratios from eight animals

Therefore, the albumin contents of the tissues were measured. These are presented in Table I, together with the ratios α_1 -microglobulin:albumin. The albumin concentrations roughly parallel the α_1 -microglobulin concentrations in all organs except the kidney and the liver. Consequently, the ratios α_1 -microglobulin:albumin in these organs are relatively constant, while higher values were obtained for the kidney and the liver. The ratio was calculated for each animal separately, before computing the mean values shown in Table I. No marked difference in the tissue distribution of α_1 -microglobulin was seen between the three solubilization techniques.

α_1 -microglobulin concentrations in subcellular fractions

Table II shows the subcellular distribution of α_1 -microglobulin in the kidney, liver, placenta, and muscle, after treatment of the subcellular fractions with 1% nonidet P-40. The values are compared to the corresponding data for albumin.

Table II

Subcellular distribution of α_1 -microglobulin and albumin

Tissue	Subcellular fraction*	% of total activity [†]	
		α_1 -microglobulin	Albumin
Kidney	I	2.2 (0.6)	1.9 (0.5)
	II	5.3 (1.3)	2.3 (0.3)
	III	3.5 (0.4)	2.5 (0.5)
	IV	89.0 (1.6)	93.3 (0.9)
Liver	I	4.0 (1.2)	4.3 (1.2)
	II	3.5 (0.7)	2.9 (1.0)
	III	25.0 (4.0)	6.3 (1.0)
	IV	67.5 (4.2)	86.5 (1.6)
Placenta	I	5.5 (0.6)	5.9 (0.2)
	II	4.0 (0.1)	5.0 (0.2)
	III	2.3 (0.2)	2.9 (0.2)
	IV	88.2 (0.4)	86.2 (0.3)
Muscle	I	N.D.	N.D.
	II	-	0.7 (0.3)
	III	-	0.8 (0.3)
	IV	100.0 (0.0)	98.5 (0.6)

- * I: 1,000 x G precipitate
II: 10,000 x G precipitate
III: 105,000 x G precipitate
IV: 105,000 x G supernatant

[†] Values are means of eight samples (S.D.)

Virtually all α_1 -microglobulin in the kidney, placenta and muscle was found in the 105,000 x G supernatants (89%, 88%, and 100%, respectively). On the other hand, 25% of the α_1 -microglobulin present in the liver was precipitated in the microsomal fraction (fraction III), while two-thirds (67.5%) remained in the supernatant after centrifugation at 105,000 x G. The albumin was almost exclusively found in the soluble fraction (fraction IV) in the kidney, liver, placenta, and muscle (93.3%, 86.5%, 86.2%, and 98.5%, respectively). The α_1 -microglobulin and albumin contents in the 1,000 x G precipitate of the muscle homogenate were not determined, since its high content of interstitial material made it difficult to solubilize with 1% nonidet P-40. Total yields of α_1 -microglobulin and albumin per gram tissue, with this method, were always comparable to those presented in Table I.

Release of α_1 -microglobulin from purified liver cells

Table III shows the amounts of α_1 -microglobulin in the media of guinea pig liver cells, after incubations for 10 min. or 4 hours. The concentration of α_1 -microglobulin increases from 10 ng per 10^6 cells to 272 ng per 10^6 cells during the four hours. In contrast, only 17 ng per 10^6 cells were released after four hours when 1.5 mM NaN_3 was present in the medium.

Table III

Release of α_1 -microglobulin from liver cells

Time of incubation	α_1 -microglobulin (ng/ 10^6 cells $\pm$ S.D.)	
	-	1.5 mM NaN_3
10 min.	10 (2)	8 (3)
240 min.	272 (28)	17 (5)

Concentrated incubation media were chromatographed on a Sephadex G-200 column. The result is shown in Figure 2. The elution volume of urinary α_1 -microglobulin is marked by an arrow. As evident from the figure, liver α_1 -microglobulin was eluted somewhat earlier than urinary α_1 -microglobulin.

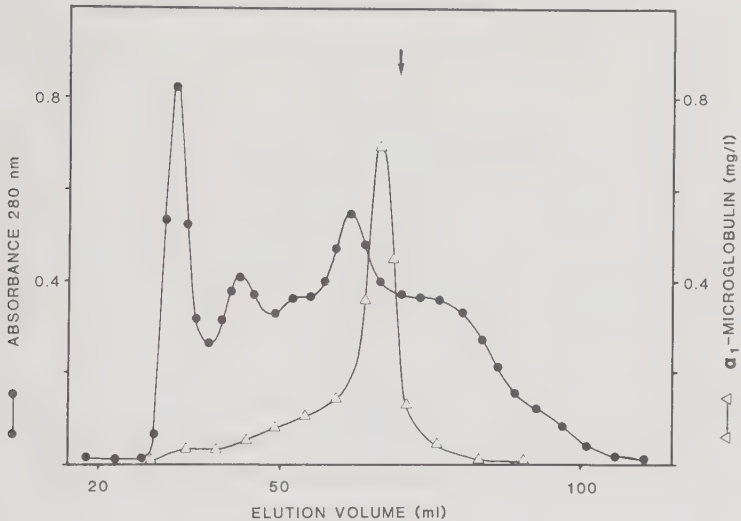


Fig.2. Gel chromatography on a column packed with Sephadex G-200 (1.1 x 100 cm) of concentrated medium from guinea pig liver cells, incubated four hours. The column was eluted at a flow-rate of 1.9 ml/hour with 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. Fractions of 0.8 ml were collected. α_1 -microglobulin concentrations were determined by radioimmunoassay.

DISCUSSION

We have investigated the tissue distribution of α_1 -microglobulin in the guinea pig by homogenization of a number of organs, followed by chemical (deoxycholate) or mechanical (sonication) solubilization of the homogenates. Greater amounts of α_1 -microglobulin were found in the liver and the kidney than in the other organs; approximately five times higher concentrations in the kidney than in the liver (Table I). The serum level of α_1 -microglobulin in the guinea pig has previously been determined to 26 mg/ml (Åkerström and Berggård, 1979), and it is obvious that a successful removal of serum from the organs by wash-out perfusion is critical to the evaluation of the data presented in Table I. To obtain an estimate of remaining blood in the tissues after the perfusion, we analysed the albumin concentrations in the tissues. Assuming that the detected albumin in the organs solely originate from the blood, the relative amounts of α_1 -microglobulin in the tissues should then be presented as the ratio between α_1 -microglobulin and albumin. This further accentuates the difference between the kidney and liver, containing high levels of α_1 -microglobulin, and the other organs, containing low levels of α_1 -microglobulin. Since albumin can be expected to be found inherently in both liver (site of synthesis) and kidney (site of degradation), the ratios in these organs should probably be higher than those given in Table I.

Tejler (1978) found higher levels of α_1 -microglobulin in the plasma of fetuses (15-18 weeks) than in adults, and lower levels of the protein in cord-blood from new-born than in maternal blood. In relation to this, it seemed logical to include the placenta in the studies of the tissue distribution of guinea pig α_1 -microglobulin. However, the ratio α_1 -microglobulin:albumin in the placenta did not differ significantly from any other of the investigated organs, apart from the kidney and the liver.

These results suggest that the liver is the site of synthesis and that kidney α_1 -microglobulin represent plasma α_1 -microglobulin undergoing glomerular filtration and renal degradation. It would be consistent with current knowledge of plasma protein metabolism. To shed some light on this hypothesis, we fractionated kidney, liver, placenta and muscle cells by homogenization and differential centrifugation. 25% of liver α_1 -microglobulin were found in the 105,000 x G precipitate, the fraction enriched in subcellular structures participating in protein synthesis. Negligible amounts were found in the corresponding fractions of kidney, placenta, and muscle. In contrast, almost all kidney α_1 -microglobulin was localized to the cytoplasmatic fraction. The high amounts of albumin in the 105,000 x G supernatants support the assumption that most of the albumin detected in these organs originate from blood.

α_1 -microglobulin was accumulated in the medium of purified guinea pig liver cells (Table III), supporting the hypothesis that hepatocytes synthesize α_1 -microglobulin. The accumulation was inhibited by the addition of sodium azide to the medium, suggesting that the α_1 -microglobulin release is an energy-requiring process.

Takagi *et al.*, (1979) used anti-human α_1 -microglobulin to study the distribution of α_1 -microglobulin in human tissue with the immunofluorescence technique. The antiserum failed to stain parenchymal cells of the liver and kidney. Svensson and Ravnkov (1976) report normal plasma levels of α_1 -microglobulin in humans with liver cirrhosis. On the other hand, significantly decreased plasma concentrations of the protein in cirrhotic patients were found by Tejler (1978) and Takagi *et al.* (1980). Moreover, Tejler *et al.* (1978) have demonstrated a synthesis of α_1 -microglobulin in human fetal liver explants. These data have been discussed elsewhere (Berggård *et al.*, 1980), and the opinion that α_1 -microglobulin is synthesized by the liver is further supported by the results presented in this work.

α_1 -microglobulin from the liver cell media appeared slightly larger than urinary α_1 -microglobulin, since it was eluted at a somewhat earlier position upon a Sephadex G-200 gel-chromatography (Figure 2). It seems that one or more determinants are hidden, altered or absent from the α_1 -microglobulin in the liver homogenate, since it could not inhibit the binding of more than approximately three-fourths of ^{125}I -labeled urinary α_1 -microglobulin (Figure 1). This suggests that the protein undergoes a physical alteration on its way between the liver and the urine.

It has been postulated that low molecular weight plasma proteins, such as α_1 -microglobulin, are filtrated in glomeruli, reabsorbed and catabolized by renal tubules (*cf.* Strober and Waldmann, 1974). The urinary excretion of α_1 -microglobulin is elevated when tubular function is impaired (Ekström and Berggård, 1977, Åkerström and Berggård, 1979). Hence, it is reasonable to assume that the α_1 -microglobulin found in the solubilized kidneys represent α_1 -microglobulin that has been filtrated and reabsorbed by the renal tubules.

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REFERENCES

- Åkerström, B. and Berggård, I. (1979) Guinea pig α_1 -microglobulin. Isolation and properties in comparison with human α_1 -microglobulin. *Eur. J. Biochem.* 101, 215-223.
- Åkerström, B., Nilsson, K., Berggård, B. and Berggård, I. (1979) In search of α_1 -microglobulin on the lymphocyte surface. *J. Immunol.* 122, 2526-2520.
- Avrameas, S. and Ternynck, T. (1969) The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry* 6, 53-66.
- Berggård, B., Ekström, B. and Åkerström, B. (1980) α_1 -microglobulin. *Scand. J. clin. Lab. Invest.* 40 (Suppl. 154) 63-71.
- Berggård, I. (1961) Proteins, glycoproteins and mucopolysaccharides in normal human urine. *Ark. Kemi* 18, 291-313.
- Björck, L., Åkerström, B. and Berggård, I. (1979) Occurrence of β_2 -microglobulin in mammalian lymphocytes and erythrocytes. *Eur. J. Immunol.* 9, 486-490.
- Böyum, A. (1968) Separation of leukocytes from blood and bone-marrow. *Scand. J. clin. Lab. Invest.* 21 (Suppl. 97) 9-29.
- Ekström, B. and Berggård, I. (1977) Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J. Biol. Chem.* 252, 8048-8057.
- Ekström, B., Peterson, P.A. and Berggård, I. (1975) A urinary and plasma α_1 -glycoprotein of low molecular weight: Isolation and some properties. *Biochem. Biophys. Res. Commun.* 65, 1427-1433.
- Greenwood, F.C. and Hunter, W.M. (1963) The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114-123.
- Hanks, J.H. and Wallace, R.E. (1949) Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71, 196-200.

- Itoh, Y., Kin, K., Kasahara, T., Sakurabayashi, I., Kawai, T., Shiori-Nakano, K. and Takagi, K. (1979) Synthesis and secretion of α_1 -microglobulin by human lymphocytes. *Clin. exp. Immunol.* 37, 134-139.
- Lögdberg, L. and Åkerström B. (1981) Immunosuppressive properties of α_1 -microglobulin. *Scand. J. Immunol.* 13, 383-390.
- Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2, 235-254.
- Marchalonais, J.J. (1969) An enzymatic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113, 299-305.
- Nilsson, Å., Sundler, R. and Åkesson, B. (1973) Biosynthesis of fatty acids and cholesterol in isolated rat liver parenchymal cells. Effect on albumin-bound fatty acids. *Eur. J. Biochem.* 39, 613-620.
- Pearlstein, E., Turesson, I., Tejler, L. and Grubb, A.O. (1977) Expression of protein HC on the plasma membrane of different human cell types. *J. Immunol.* 119, 824-829.
- Peterson, P.A. and Berggård, I. (1971) Isolation and properties of a human retinol-transporting protein. *J. Biol. Chem.* 246, 25-33.
- Plesner, T., Nørgaard-Pedersen, B. and Boenisch, T. (1975) Radioimmunoassay of β_2 -microglobulin. *Scand. J. clin. Lab. Invest.* 35, 729-735.
- Seglen, P.O. (1973) Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exptl. Cell. Res.* 82, 391-398.
- Strober, W. and Waldmann, T.A. (1974) The role of the kidney in the metabolism of plasma proteins. *Nephron* 13, 35-66.
- Svensson, L. and Ravnskov, U. (1976) α_1 -microglobulin, a new low molecular weight plasma protein. *Clin. Chim. Acta* 73, 415-422.
- Takagi, K., Itoh, Y., Enomoto, H., Koyamaishi, Y., Maeda, K. and Kawai, T. (1980) A comparative study of serum α_1 -microglobulin and β_2 -microglobulin levels in cancerous and other diseases. *Clin. Chim. Acta* 108, 277-283.

- Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T. and Shikata, T. (1979) Tissue distribution of human α_1 -microglobulin. *J. clin. Invest.* 63, 318-325.
- Tejler, L. (1978) Protein HC. A low molecular weight plasma protein. *Ph.D. Thesis*. University of Lund, Sweden.
- Tejler, L., Eriksson, S., Grubb, A. and Åstedt, B. (1978) Production of protein HC by human fetal liver explants. *Biochim. Biophys. Acta* 542, 506-514.
- Tejler, L. and Grubb, A.O. (1976) A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine and cerebrospinal fluid. *Biochim. Biophys. Acta* 439, 82-94.

SYNTHESIS OF α_1 -MICROGLOBULIN BY GUINEA PIG LIVER

by

Bo Åkerström

SUMMARY

Explants from perfused guinea pig livers were found to release α_1 -microglobulin into the culture medium. Tritiated leucine in the medium was incorporated into the protein, suggesting a *de novo* synthesis of α_1 -microglobulin by the liver tissue. The size of the protein could not be distinguished from that of purified urinary α_1 -microglobulin when tested with sodium dodecyl sulfate-polyacrylamide gel-electrophoresis. After intravenous injections of tritiated leucine into guinea pigs, the 105,000 x G pellet of homogenized liver rapidly increased its content of radioactive α_1 -microglobulin, with a maximum after 20 minutes. [^3H] α_1 -microglobulin appeared in serum after a lag-phase of 20 minutes, and by comparing the rate of accumulation with albumin, the synthesis of guinea pig α_1 -microglobulin could be estimated to 20 $\mu\text{g/g}$ liver and hour.

INTRODUCTION

α_1 -microglobulin is a plasma glycoprotein isolated from the urine of human patients with tubular dysfunction (Ekström *et al.*, 1975, Ekström and Berggård, 1977). It has a molecular weight of 26,000, binds a brown substance of unknown structure (Ekström and Berggård, 1977), and consists of molecular subpopulations with varying net charge (Åkerström and Berggård, 1979, Vincent *et al.*, 1982). It is present in human plasma at 30-100 mg/l in monomeric form as well as in complex with other plasma proteins (Ekström *et al.*, 1975). Lögdberg and Åkerström (1981) have demonstrated immunoregulatory properties of human α_1 -microglobulin. Production of α_1 -microglobulin by lymphocytes have been reported (Tejler *et al.*, 1976, Takagi *et al.*, 1979), but we have shown that these results are probably due to contaminants in the α_1 -microglobulin preparations (Åkerström *et al.*, 1979). Tejler *et al.* (1978) demonstrated a production of α_1 -microglobulin by human fetal liver explants. Takagi *et al.* (1979) presented opposing results from studies with human liver sections. Thus, the origin and localization of α_1 -microglobulin is still unclear.

The work presented in this report was undertaken to clarify the role of liver in the synthesis of α_1 -microglobulin.

MATERIALS AND METHODS

Proteins and antisera

Guinea pig α_1 -microglobulin (Åkerström and Berggård, 1979) and guinea pig albumin were purified in this laboratory. Albumin was isolated from 20 ml guinea pig serum by zone electrophoresis (Peterson and Berggård, 1971) in 0.1 M barbital buffer, pH 8.6, using Pevikon C-870 (Kema Nord AB, Stockholm, Sweden) as supporting medium, followed by gel-chromatography on a Sephadex G-200 column (140 x 5 cm), equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. Rabbit antisera to guinea pig α_1 -microglobulin and albumin were prepared as previously described (Åkerström and Berggård, 1979). Rabbit

anti-guinea pig α_1 -microglobulin was absorbed with glutaraldehyde-insolubilized guinea pig serum (Avrameas and Ternynck, 1969), depleted of monomeric α_1 -microglobulin by gel filtration. Guinea pig β_2 -microglobulin, rabbit anti-guinea pig β_2 -microglobulin were prepared according to Cig n *et al.* (1978), and goat anti-rabbit IgG as outlined by Bj rck *et al.* (1977).

Liver cultures

Outbred guinea pigs, 700-1100 g. were anesthetized with intraperitoneally injected Mebumal ^(R) (Aco, Sweden), 45 mg/kg body weight. The liver was perfused *in situ* through vena porta (outflow through the vena cava inferior) for 2 minutes with an oxygenated calcium free Hanks' solution (Hanks and Wallace, 1949), substituting the bicarbonate and phosphate with 40 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (Hepes) (Flow Laboratories, Ltd., Irvine, Scotland), at 37 C and a flow rate of approximately 25 ml/minute. Randomly chosen pieces were immediately cut from the liver, and moistened with a few drops of medium. Cultures were prepared according to Aspegren and Danielsson (1974) with some modifications. The liver was cut into small explants (approximately 3 x 3 x 1.5 mm), and placed on a strip of millipore filter (0.45 μ , Millipore Inc., Bedford, England), which in turn was put on a slice of cellulose acetate fabric (Spongostan ^(R), Ferrosan, Copenhagen). Three such liver explants were placed in a closed cylindrical glass vial (I.D. 43 mm) with 4 ml medium. The cultures were prepared at room-temperature, and were completed within fifteen minutes. Incubation of cultures was performed at 37 C. L-15 medium (Flow Laboratories Ltd.) was used, supplemented with 1 mM sodiumsuccinate, 29 mM Hepes, porcine insulin (0.5 mg/l, Novo Industri, Bagsvaerd, Denmark), 2% fetal calf serum (FCS), and antibiotics (penicillin, 100 mg/l, and gentamycin 50 mg/l), at pH 7.4. Labelling was done with tritiated leucine (L-[4,5-³H]leucine, specific activity 136 Ci/mmol, in water solution, 1.0 mCi/ml, code TRK. 510 (The Radiochemical Centre, Amersham, England). Liver explants were precultured for four hours with the supplemented L-15 medium, depleted of leucine, the medium was discarded and replaced with fresh leucine-free medium,

containing tritiated leucine at 0.1 mCi/ml. The medium was harvested after 48 hours, immediately frozen at -20°C , and replaced with fresh $[^3\text{H}]$ leucine-L-15 medium.

After 5-9 days of culture, liver explants were examined under microscope after fixation, embedding in paraffin, slicing, and staining. On the 8-9th day, a beginning degeneration of the liver parenchyme could be seen in the interior parts of the explants.

In vivo incubations with $[^3\text{H}]$ leucine

Outbred guinea pigs, 500-600 g, were anesthetized as above. 0.5 mCi $[^3\text{H}]$ leucine per kg body weight was injected in the abdominal vena cava. At desired times blood was collected from the heart, and two pieces were taken from the liver. The liver sections were immediately frozen until further analyzed. The blood was allowed to clot, and the serum frozen. The weight of the liver from the guinea pigs in these experiments was approximately 5% of the body weight.

Fractionation of liver

The liver explants were homogenized in ice-cold 50 mM Tris-HCl, pH 8.5 + 0.25 M sucrose and 1 mM EDTA and diisopropyl-phosphor-fluoridate (DFP), 2 ml/3 explants or 5 ml/2 g liver, with a Potter-Elvehjem loose-fitting teflon pestle (seven strokes). The suspensions were centrifuged at $10,000 \times G$ for 30 minutes at 4°C . The precipitates were suspended in 2 ml ice-cold Tris-sucrose buffer, and the supernatants were transferred to a new set of tubes, and centrifuged at $105,000 \times G$ for 60 minutes at 4°C . These precipitates were suspended in 1 ml ice-cold Tris-sucrose buffer. Nonidet P-40 (NP-40) was added to 0.5% to the $10,000 \times G$ precipitate, the $105,000 \times G$ precipitate, and to the $105,000 \times G$ supernatant. After 10 minutes at 4°C , all fractions were centrifuged at $105,000 \times G$ for 60 minutes at 4°C , and the supernatants were frozen until further analysis. Total protein was prepared by adding 4 volumes of 12.5% trichloroacetic acid (TCA) to specimens of liver homogenates or

serum, and washing the precipitates with 5% TCA. The pellets were dissolved in 0.3 M NaOH. α_1 -microglobulin or albumin was isolated by specific immunoprecipitation of liver fractions and serum, pretreated with rabbit anti-guinea pig β_2 -microglobulin and goat anti-rabbit IgG. Counting efficiency of ^3H was estimated with internal standards.

Radioimmunoassays

α_1 -microglobulin and albumin were determined with the radioimmunoassay described by Plesner *et al.* (1975), using whole antiserum diluted to a binding capacity of 50% of the labelled α_1 -microglobulin or albumin. Standards, antisera, radiolabelled protein, and unknown samples were diluted with 0.1 M phosphate buffer, pH 7.5 with 0.1% bovine serum albumin (BSA) and 0.02% NaN_3 . α_1 -microglobulin and albumin were radioiodinated with ^{125}I (The Radiochemical Centre, Amersham, England) by the chloramin-T method (Greenwood and Hunter, 1963), or with lactoperoxidase (Marchalonais, 1969), using a radioiodination kit (New England Nuclear, Boston, USA).

Immunoprecipitation

Immunoprecipitation of radiolabelled antigen with rabbit-antisera and goat anti-rabbit IgG was performed as described earlier (Åkerström *et al.*, 1979), centrifugating the precipitates through a discontinuous sucrose gradient as outlined by Taylor and Schimke (1973). The immunoprecipitates were analysed for ^3H or ^{125}I , or subjected to sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE).

SDS-PAGE

SDS-PAGE was done according to Neville (1971). Separation gels had a total acrylamide concentration of 13.7% and the degree of cross-linking was 3.7%. When analysing tritiated samples, the gels were sliced into 1 mm-pieces, to each of which were added 3 ml of the liquid scintillation system Lumasolve-Lipoluma (Lumac Systems A.G., Basel, Switzerland).

Other methods

Concentration of protein solutions were accomplished by ultra-filtration with 24/32 in Visking dialysis tubing (Union Carbide Corp., USA) as described elsewhere (Berggård, 1961).

RESULTS

Accumulation of α_1 -microglobulin in liver culture medium

α_1 -microglobulin, analyzed by radioimmunoassay, was accumulated in the culture medium of liver explants. Figure 1 shows the concentrations of α_1 -microglobulin within 48 hours of incubation. In these experiments the medium was not changed during the two days. A continuously rising α_1 -microglobulin concentration was seen, and approximately 30 ng α_1 -microglobulin/ 10^6 cells were found at the end of the incubation. A similar increase of the albumin concentrations was measured (Figure 1, dashed line), although this protein displayed a somewhat more rapid increase during the first hours.

Concentrated incubation media were chromatographed on a Sephadex G-200 column. The result is shown in Figure 2. The elution volume of α_1 -microglobulin in guinea pig serum is marked with an arrow. As evident from the figure, most of the liver α_1 -microglobulin was eluted at the same position as serum α_1 -microglobulin.

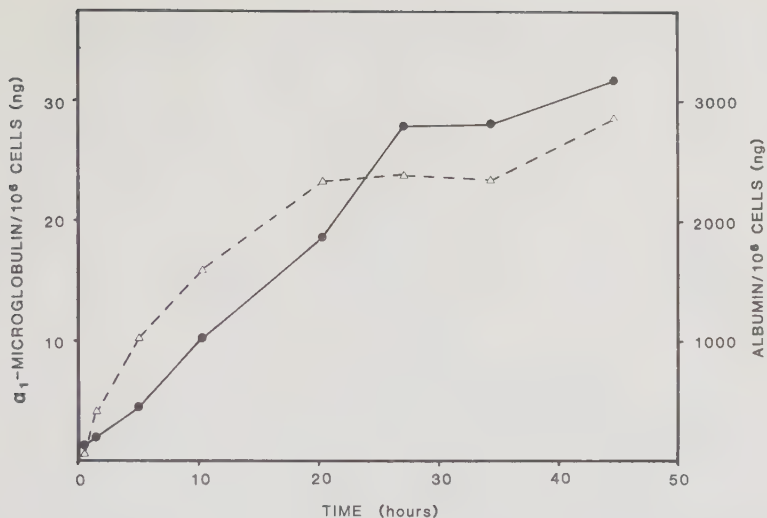


Fig. 1. Amount of α_1 -microglobulin (●—●) and albumin (△—△)/10⁶ cells, in the medium during the first 48 hours of incubation of three liver explants (~30 x 10⁶ cells) in 4 ml medium, expressed as ng/10⁶ cells.

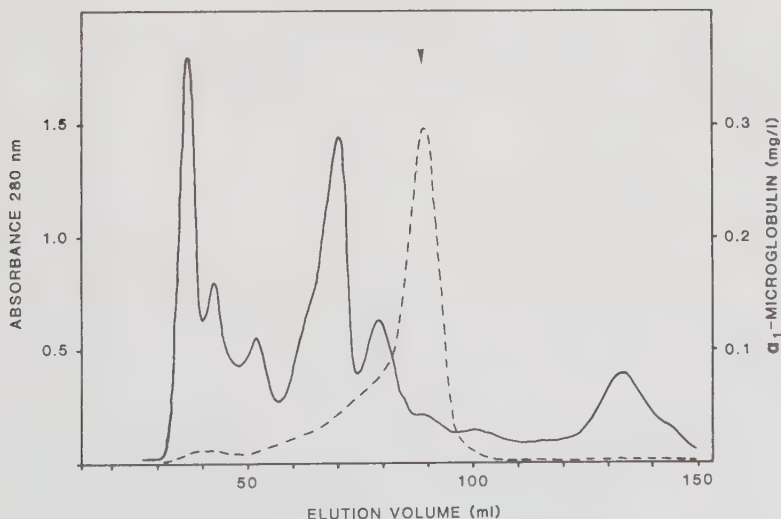


Fig. 2. Gel chromatography on Sephadex G-200 of concentrated medium from liver explant incubations. The column (1.1 x 100 cm) was equilibrated with 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 0.02% NaN₃, and eluted with a flow-rate of 1.9 ml/hour. The fractions were analysed for α_1 -microglobulin (----) by radio-immunoassay. The position of monomeric guinea pig α_1 -microglobulin in serum is marked by an arrow.

De novo synthesis of α_1 -microglobulin by liver explants

[^3H]leucine, added to the culture medium, was incorporated to a non-dialyzable and TCA-precipitable fraction. The media from three successive 48-hour incubations with [^3H]leucine were pooled, and from that 0.7% (0.5-1.0%) of the radioactivity was retained upon ultrafiltration, and 0.4% (0.3-0.5%) could be precipitated by 12.5% TCA. After concentration and dialysis of the pooled media, it was applied to a Sephadex G-200 column, and the radioactivity was eluted as shown in Figure 3.

Fractions were pooled as illustrated in the figure, according to previous calibration experiments determining the elution volume of α_1 -microglobulin in guinea pig serum. Immunoprecipitation of the pooled fractions with anti- α_1 -microglobulin, followed by separation of the bound radioactive material with SDS-PAGE, gave the result shown in Figure 4. A major peak with a migration distance corresponding to purified guinea pig α_1 -microglobulin was found. Analysis of material bound to anti-albumin yielded a peak with the size of albumin (Figure 4).

Both antisera precipitated an additional, minor peak, at a position corresponding to 44-46,000 daltons.

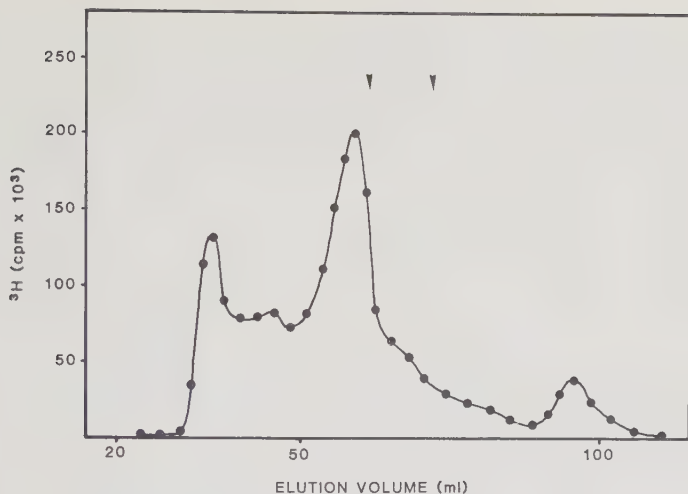


Fig. 3. Gel chromatography on Sephadex G-200 of the concentrated and dialyzed medium from three successive 48-hour incubations of nine liver explants, grown with [^3H]leucine added to the medium. The column (1.1 $\times$ 80 cm) was equilibrated with 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.02% NaN_3 , and eluted at a flow-rate of 1 ml/hour. Fractions between the arrows were pooled and subjected to further studies.

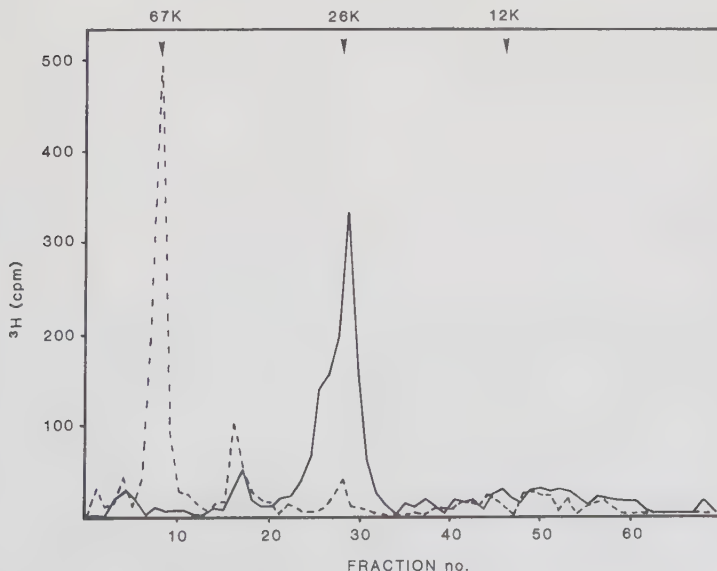


Fig. 4. SDS-PAGE of medium from three successive 48-hour incubations of nine liver explants, grown with [^3H]leucine in the medium, gel-filtrated as shown in Fig 3, and precipitated with anti- α_1 -microglobulin (—), and anti-albumin (---).

In vivo incorporation of [^3H]leucine

After injection into the blood-stream of guinea pigs, [^3H]leucine was rapidly eliminated from the blood. Figure 5 shows the content of [^3H]leucine in the blood, and in liver- and blood protein, after intravenous injection. After five minutes, approximately 6% of the injected [^3H]leucine still remained in the blood and after 30 minutes 1% was left. The incorporation of the radioactivity into the liver protein was maximal after 20-30 minutes. At that time 23% of the injected radioactive dose was found in the liver protein. The [^3H]leucine content of serum proteins increased continuously after a lag-phase of approximately 20 minutes.

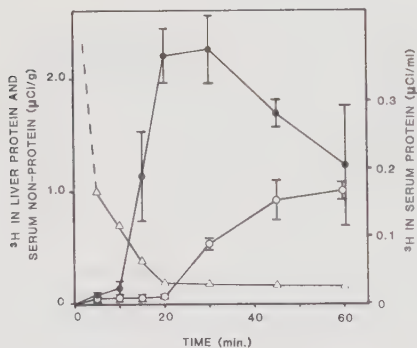


Fig. 5. [^3H]leucine in blood (Δ — Δ), liver protein ($\bullet$ — $\bullet$), and blood protein ($\circ$ — $\circ$), after injection of 0.5 mCi [^3H]leucine/kg guinea pig. Each point represents the mean $\pm$ S.D. of two animals.

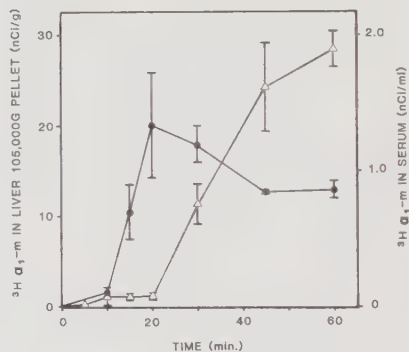


Fig. 6. [^3H] α_1 -microglobulin in liver 105,000 $\times$ G pellet ($\bullet$ — $\bullet$), and serum (Δ — Δ), after injection of 0.5 mCi [^3H]leucine/kg guinea pig. Each point represents the mean $\pm$ S.D. of two animals.

Immunoprecipitation of the radioactivity in liver with anti- α_1 -microglobulin revealed rapidly increasing amounts of [^3H]- α_1 -microglobulin in the 105,000 $\times$ G pellet of liver homogenate (Figure 6). A maximum was reached 20 minutes after the injection. In serum, virtually no tritiated α_1 -microglobulin was detected the first 20 minutes. After that time a steady increase was displayed as shown in Figure 6. The immunoprecipitated, radioactive material appeared as one component with an apparent molecular weight of 26,000 upon SDS-PAGE. The appearance of [^3H]albumin was analysed, and is compared to [^3H] α_1 -micro-

globulin in Figure 7. Between 20 and 30 minutes the rate of incorporation of radioactivity into albumin was 34 times that of α_1 -microglobulin. After 60 minutes, albumin carried 24% and α_1 -microglobulin 1.1% of the total radioactivity found in serum proteins.

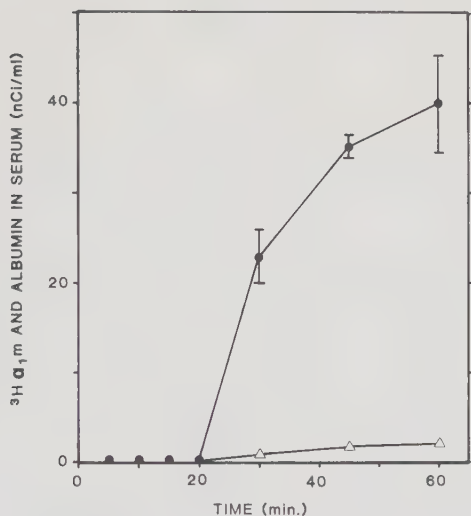


Fig. 7. [³H] α_1 -microglobulin (Δ — Δ), and [³H]albumin ($\bullet$ — $\bullet$) in serum, after injection of 0.5 mCi [³H]leucine/kg guinea pig. Each point represents the mean $\pm$ S.D. of two animals.

DISCUSSION

Media of organ cultures from guinea pig livers were shown to contain material reacting with antiserum against α_1 -microglobulin purified from guinea pig urine. [³H]leucine, when added to the culture medium, was incorporated into the anti- α_1 -microglobulin reacting material. Gel-chromatography experiments as well as SDS-PAGE demonstrate that the size of the α_1 -microglobulin antigen from liver explant media is similar to that of urinary α_1 -microglobulin. These results strongly suggest that the antigen from guinea pig liver explant medium is α_1 -microglobulin, and that it is synthesized by the liver tissue. It is highly improbable that a selective, non-specific binding

is responsible for the precipitation of a tritiated antigen with anti- α_1 -microglobulin, since anti-albumin bound another antigen with the size of albumin. This conclusion is also supported by the findings presented in Figure 6. Here, newly synthesized α_1 -microglobulin is accumulated in the $105,000 \times G$ pellet of the liver during the first 20 minutes after injection of [3H]leucine. The α_1 -microglobulin is not transported to the liver, since it can not be found in serum until after 20 minutes.

The heterogeneity of the α_1 -microglobulin peak, analysed by SDS-PAGE (Figure 4), suggests the presence in the culture medium of an α_1 -microglobulin-species with a higher molecular weight. This component is interesting as a possible candidate for a pro-form of α_1 -microglobulin. No larger component has been found in guinea pig serum (unpublished observations) and urine (Åkerström and Berggård, 1979). Another molecule, with an apparent size of 44-46,000 daltons, was precipitated by anti-albumin and anti- α_1 -microglobulin. As has been discussed by Berggård *et al.* (1980), minor impurities of anti- α_1 -microglobulin sera can be present in this range of molecular weights. This, and the fact that the component is precipitated by both antisera, suggest that it should be regarded as an impurity or an artefact. The behaviour of α_1 -microglobulin in liver incubation media on gel-chromatography (Figure 2) can be compared to α_1 -microglobulin in guinea pig serum (Åkerström and Berggård, 1979). The elution profile of guinea pig serum includes an α_1 -microglobulin-peak at approximately 200,000 daltons, corresponding to the complex with IgA, described for human α_1 -microglobulin (Tejler and Grubb, 1976). No complex IgA- α_1 -microglobulin is present in liver medium. As expected, the binding of α_1 -microglobulin to IgA seems to be an extra-hepatic process.

The biosynthesis of α_1 -microglobulin was also studied under *in vivo* conditions, by intravenously injecting [3H]leucine in anesthetized guinea pigs. By measuring TCA-non-precipitable radioactivity in serum, it could be shown that 94% of the injected dose had been removed from the blood within five

minutes, and after 20 minutes only 1% remained in the blood. Thus, the effect was that of a pulse experiment. The pathway of [^3H]leucine, as deduced from Figure 5, was: free [^3H]leucine in blood $\rightarrow$ protein in liver $\rightarrow$ protein in blood. This is in agreement with basic metabolic concepts, and therefore the experimental system apparently worked. The tracer-dose of [^3H]leucine was 3.7 nanomoles per kg guinea pig. This amounted to a 80 nM- or less than 0.1% increase of the expected concentration of leucine in serum. Figure 6 clearly indicates a synthesis of α_1 -microglobulin by the liver. [^3H] α_1 -microglobulin was rapidly accumulated in the 105,000 x G pellet of homogenized liver, reached a maximum and started to decline concurrently with its first appearance in serum. The rate of incorporation of tritium into serum albumin between 20 and 30 minutes was 34 times that of α_1 -microglobulin. Rat serum albumin has been reported by Peters and Peters (1972) to be synthesized at 0.34 mg/g liver and hour, and Kelman *et al.* (1972) estimated the synthesis of human serum albumin to 0.51 mg/g liver and hour. Assuming a synthesis of guinea pig serum albumin at 0.4 mg/g liver and hour, it can be estimated that guinea pig α_1 -microglobulin is synthesized at a rate of 20 ($\pm$ 8) μg α_1 -microglobulin/g liver and hours. For the guinea pigs of 550 g used in this study, this means 12 ($\pm$ 5) mg/day. Åkerström and Berggård (1979) have determined the normal daily excretion or urinary α_1 -microglobulin for guinea pigs to 1.4 mg, *i.e.* 12% of the rate of synthesis. As discussed by Strober and Waldmann (1974), this figure has been reported to be less than 1% for low-molecular weight plasma-proteins in rats and humans. However, observations in our laboratory indicate notable urinary excretions of albumin, α_1 -microglobulin, retinol-binding protein and β_2 -microglobulin among untreated guinea pigs (B. Åkerström, L. Björck, R. Cigén, and L. Lögdberg, unpublished observations).

Human fetal liver, a major site of hematopoiesis, has been shown to synthesize α_1 -microglobulin (Tejler *et al.*, 1978). Opposing results were reported by Takagi *et al.* (1979), who failed to stain human liver sections with anti- α_1 -microglobulin. The work presented here, demonstrates the synthesis of α_1 -micro-

globulin by adult guinea pig liver, and thus seems to connect the production of α_1 -microglobulin with the hepatic, rather than the hematopoietic function of the fetal liver. However, other cell-types than the hepatocyte are present in the liver, and more work will have to be done to find out which of these are responsible for the production of α_1 -microglobulin.

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REFERENCES

- Åkerström, B. and Berggård, I. (1979) Guinea pig α_1 -microglobulin. Isolation and properties in comparison with human α_1 -microglobulin. *Eur. J. Biochem.* 101, 215-223.
- Åkerström, B., Nilsson, K., Berggård, B. and Berggård, I. (1979) In search of α_1 -microglobulin on the lymphocyte surface. *J. Immunol.* 122, 2516-2520.
- Aspegren, K. and Danielsson, H. (1974) Growth quantitation of human mammary carcinoma in organ tissue culture. *Am. J. Surg.* 128, 42-48.
- Avrameas, S. and Ternynck, T. (1969) The crosslinking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry* 6, 53-66.
- Berggård, B., Ekström, B. and Åkerström, B. (1980) α_1 -microglobulin. *Scand. J. clin. Lab. Invest.* 40, Suppl. 154, 63-71.
- Berggård, I. (1961) Proteins, glycoproteins and mucopolysaccharides in normal human urine. *Ark. Kemi* 18, 291-313.
- Björck, L., Cigén, R., Berggård, B., Löw, B. and Berggård, I. (1977) Relationships between β_2 -microglobulin and alloantigens coded for by the major histocompatibility complexes of the rabbit and the guinea pig. *Scand. J. Immunol.* 6, 1063-1069.
- Cigén, R., Ziffer, J.A., Berggård, B., Cunningham, B.A. and Berggård, I. (1978) Guinea pig β_2 -microglobulin. Purification, properties, and partial structure. *Biochemistry* 17, 947-955.
- Ekström, B. and Berggård, I. (1977) Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J. Biol. Chem.* 252, 8048-8057.
- Ekström, B., Peterson, P.A. and Berggård, I. (1975) A urinary and plasma α_1 -glycoprotein of low molecular weight: Isolation and some properties. *Biochem. Biophys. Res. Commun.* 65, 1427-1433.
- Greenwood, F.C. and Hunter, W.M. (1963) The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114-123.

- Hanks, J.H. and Wallace, R.E. (1949) Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71, 196-200.
- Kelman, L., Saunders, S.J., Frith, L., Wicht, S. and Corrigan, A. (1972) Effects of dietary protein restriction on albumin synthesis, albumin catabolism, and the plasma aminogram. *Am. J. Clin. Nutr.* 25, 1174-1178.
- Lögdberg, L. and Åkerström, B. (1981) Immunosuppressive properties of α_1 -microglobulin. *Scand. J. Immunol.* 13, 383-390.
- Marchalonis, J.J. (1969) An enzymatic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113, 299-305.
- Neville, D.M., Jr. (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* 246, 6328-6334.
- Peters, T., Jr. and Peters, J.C. (1972) The biosynthesis of rat serum albumin. VI. Intracellular transport of albumin and rates of albumin and liver protein synthesis *in vivo* under various physiological conditions. *J. Biol. Chem.* 247, 3858-3863.
- Peterson, P.A. and Berggård, I. (1971) Isolation and properties of a human retinol-transporting protein. *J. Biol. Chem.* 246, 25-33.
- Plesner, T., Nørgaard-Pederson, B. and Boenisch, T. (1975) Radioimmunoassay of β_2 -microglobulin. *Scand. J. clin. Lab. Invest.* 35, 729-735.
- Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T. and Shikata, T. (1979) Tissue distribution of human α_1 -microglobulin. *J. clin. Invest.* 63, 318-325.
- Taylor, J.M. and Schimke, R.T. (1973) Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. *J. Biol. Chem.* 248, 7661-7668.
- Tejler, L., Eriksson, S., Grubb, A. and Åstedt, B. (1978) Production of protein HC by human fetal liver explants. *Biochim. Biophys. Acta* 542, 506-514.

- Tejler, L., Grubb, A.O. and Turesson, I. (1976) Immuno-fluorescent demonstration of the presence of protein HC on the surface of human lymphocytes. *Acta Med. Scand.* 199, 425-427.
- Vincent, C., Revillard, J.P., Bouic, P. and Brochier, J. (1982) Study of α_1 -microglobulin (HC Protein) with monoclonal antibodies. In *Protides of the biological fluids* (Ed. Peeters, H., Pergamon Press Ltd., Oxford, England).

IN SEARCH OF α_1 -MICROGLOBULIN ON THE LYMPHOCYTE SURFACE¹

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α_1 -Microglobulin was found by immunofluorescence not to be associated with human lymphoid and nonlymphoid cell lines. No accumulation of α_1 -microglobulin was detected in culture media of these cell lines. A weak membrane fluorescence with anti- α_1 -microglobulin on peripheral lymphocytes could not be blocked by the purified protein. No release of α_1 -microglobulin into the growth medium was seen by normal cultured leukocytes. Treatment of normal lymphocytes, erythrocytes, and various cell lines with solubilization techniques did not yield any α_1 -microglobulin. α_1 -Microglobulin and protein HC display immunologic and biochemical identity. However, anti-protein HC stained almost all of the tested cell lines and normal lymphocytes. Blocking experiments with the purified protein were not successful. Antibodies reacting with a minor impurity (50 000 d) in the α_1 -microglobulin or protein HC preparations could be absorbed from anti- α_1 -microglobulin with normal leukocytes and a lymphoid cell line.

α_1 -Microglobulin (α_1 -m),⁴ a glycoprotein originally isolated from the urine of patients with renal defects (1, 2), has a m.w. of approximately 26,000 d of which 20% consist of carbohydrate. It is electrophoretically heterogeneous and binds a brown-colored material of unknown structure. α_1 -M is present in serum and has a tendency to form aggregates in serum and urine. The function of α_1 -m is unknown and the site of synthesis is still unclear.

Reports on a seemingly identical protein called protein HC have been published by Tejler and Grubb (3). Protein HC has been reported to be present on the surface of lymphocytes, erythrocytes, and fibroblast cell lines (4, 5). Our preliminary data suggesting the absence of α_1 -m on peripheral blood cells prompted us to make a thorough investigation of the possible

association of α_1 -m with blood lymphocytes, erythrocytes, or some established lymphoid and nonlymphoid cell lines. Experiments were also performed to reveal possible antigenic dissimilarities between α_1 -m and protein HC that could explain the divergent results with the two, so far, biochemically identical proteins.

MATERIALS AND METHODS

Cells and cell cultures. Peripheral blood lymphocytes were prepared as described (6, 7). Erythrocytes were purified from the sediment of defibrinated blood through the use of a cotton wool column and three successive centrifugations on Lymphoprep, density 1.077 (Nyegaard & Co., Norway), followed by washing with PBS. Peripheral white blood cells were prepared and cultivated essentially as described (8). The cells were incubated in Parker 199 medium (SBL, Sweden), containing 20% (v/v) inactivated horse serum (Flow Laboratories, U. K.) and antibiotics (penicillin, 12 μ g/ml, and streptomycin, 8 μ g/ml) with or without PHA-M (10 μ l reconstituted PHA-M/ml) (DIFCO, Detroit, Mich.) at a concentration of 2×10^6 cells/ml at 37°C in a 5% CO₂-air atmosphere. Samples were removed at appropriate intervals during 12 days, centrifuged, and the concentrations of α_1 -m, protein HC and β_2 -microglobulin were determined in the supernatants. In one experiment the cells were cultured with or without PHA-M for three days, and the supernatants were concentrated 20 times by ultrafiltration (9) before determination of the proteins.

Cell lines. A panel of human lymphoid and nonlymphoid cell lines was employed. The types of cell line and their origin are given in Tables I and II. The lymphoid cell lines were grown as nonstirred suspension cultures in a 5% CO₂-air atmosphere at 37°C as described (10). The medium, F-10, supplemented by 10% newborn calf serum and antibiotics (100 IU/ml of penicillin, 50 μ g/ml of streptomycin, and 1.25 μ g/ml of amphotericin B), was changed twice weekly. The cell concentration in the cultures was adjusted to ensure logarithmic growth throughout the incubation period.

The nonlymphoid cell lines were cultivated as monolayers in plastic Petri dishes (Nunc, Nunc, Denmark). The incubation conditions were identical to those described above for the lymphoid cell lines. The exponentially growing cells were harvested from the Petri dishes by rubber policeman.

In experiments to study the accumulation of α_1 -m in the medium, washed cells were grown for 48 hr in the above medium at an initial cell concentration of 10^5 cells/ml. The supernatants were concentrated 25 to 90 times by ultrafiltration before quantitation of α_1 -m.

Proteins. α_1 -M and β_2 -microglobulin were purified from the urine of patients with proteinuria due to chronic cadmium poisoning, as described earlier (2, 11). Both pooled and individ-

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³ Deceased in June 1978.

⁴ Abbreviations used in this paper: α_1 -m, α_1 -microglobulin; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NaDOC, sodium-deoxycholate; FITC-Swarlg, fluorescein isothiocyanate-coupled swine anti-rabbit Ig; NaSCN, sodium thiocyanate.

ual urines were used in the α_1 -m preparations. IgG from non-immunized rabbits was obtained by chromatography on DEAE-cellulose (Serva Feinbiochemica, West Germany) as described by Björck *et al.* (12). Protein HC was given to us by Dr. Anders Grubb, Malmö.

Antisera. Antisera against α_1 -m and human β_2 -microglobulin were prepared in rabbits. The immunization procedure has been detailed elsewhere (11). Antisera were raised against α_1 -m from pooled urine as well as from urine of a single individual, and both kinds were used in all experiments. The antisera designated 82, 83, and 108 showed, in addition to a strong precipitin arc in the α_1 -region, a weak cathodal arc, when tested on immunoelectrophoresis against varying amounts of concentrated urinary proteins. Anti- α_1 -m serum 319 showed only the main precipitin arc.

Fluorescein isothiocyanate-labeled swine anti-rabbit immunoglobulin (FITC-Swarlg) was purchased from Dakopatts, Copenhagen. Control sera were obtained from nonimmunized rabbits. Goat anti-rabbit IgG was prepared as outlined by Björck *et al.* (12). Anti-protein HC and anti- α_1 -acid glycoprotein (anti-orosomucoid) were gifts from Dr. Anders Grubb, Malmö and Dr. Leif Andersson, Helsinki, respectively.

Absorption of antiserum with cells. White blood cells (10^8) were incubated with heat-inactivated normal rabbit serum for 30 min at room temperature. The cell suspension was centrifuged and the supernatant was discarded. Two microliters of anti- α_1 -m serum 108 were added to the cells after dilution to 200 μ l with PBS. After incubation for 30 min at room temperature, the cells were centrifuged and the supernatant was submitted to analysis. In one experiment anti- α_1 -m was absorbed with 10^8 cells of the lymphoid cell line NALL-1. Because these cells had been frozen and thawed once before the incubations with the sera, the centrifugations were carried out at 105,000 $\times$ G.

Solubilization of cells. The experimental procedure of Evrin and Pertoff (13) was used with minor modifications. The number of peripheral blood lymphocytes submitted to sonication were 1 to 3×10^7 , erythrocytes 1 to 3×10^8 , and cultured cell lines (NALL-1, U-698 and U-393 OS) 1×10^8 . The cells were suspended in 0.25 M sucrose. Sonication with a Branson Sonic Power Sonifier was performed twice in this medium for 10 sec. The cultured cell lines were freeze-thawed once before the sonication. Four aliquots of equal volume from each homogenate were treated with 1) HCl to pH 2.3, 2) 1% sodium-deoxycholate (NaDOC) in 0.05 M Tris-HCl, pH 8.5, 3) 3 M sodium thiocyanate (NaSCN) in 0.05 M Tris-HCl, pH 8.5, and 4) 0.05 M Tris-HCl, pH 8.5, respectively. In one experiment with erythrocytes, the four sonicated aliquots were treated with 1% NaDOC in Tris-buffer, 1% Nonidet P-40 (v/v) in Tris-buffer, 1% Triton X-100 (v/v) in Tris-buffer, and with the Tris-buffer only. All samples were left at 4°C for 15 min, after which 5 volumes of the Tris-sucrose buffer were added. The sample exposed to NaSCN was then dialyzed against 1000 volumes of the same buffer for 2 hr at 4°C.

After centrifugation at 105,000 $\times$ G for 75 min at 4°C, the supernatants were analyzed for α_1 -m and protein HC. Blind samples, composed of 0.25 M sucrose or of 0.25 M sucrose containing human serum or purified α_1 -m in bovine serum, were simultaneously submitted to the same treatment as the samples containing cells.

Immunofluorescence procedures. The lymphoid cell lines and peripheral blood lymphocytes from healthy individuals and patients with chronic lymphocytic leukemia were examined by indirect immunofluorescence as previously detailed (14, 15).

Briefly, living cells were washed three times in PBS and incubated with anti- α_1 -m or control normal rabbit serum (NRS) at 4°C with FITC-Swarlg (see footnote 4). After three final washings, the cells were mounted in glycerol:PBS (1:1) and examined in a Leitz UV-microscope equipped with a Ploem-type vertical illumination. Staining of living nonlymphoid cells, growing as sparse monolayers on cover slips, was carried out essentially as described for the lymphoid cells.

The presence of cytoplasmic α_1 -m was examined by indirect immunofluorescence on fixed cells (acetone:ethanol, 11:1, 30 min or 2% paraformaldehyde, 1 hr). Incubation conditions with the antisera were identical to those described above.

Radioimmunoassay (RIA). The RIA described by Plesner *et al.* (16) was used for all determinations of α_1 -m, protein HC, and β_2 -microglobulin. The proteins were radiolabeled with 125 I (Amersham, England) by the chloramine-T method (17). 125 I-proteins were separated from free iodide by gel chromatography on a Sephadex G-50 column (Pharmacia, Sweden). Whole antisera were used, diluted to a binding capacity of 50% of the labeled antigen.

Immunoelectrophoresis. Immunodiffusion and microimmunoelectrophoresis were carried out as described (11, 18, 19). Immunoprecipitation was accomplished by incubating radiolabeled α_1 -m or protein HC with a manifold excess of anti- α_1 -m or anti-protein HC for 30 min at 37°C and then adding a slight excess of goat anti-rabbit IgG. The incubations were carried out in a 0.05 M phosphate buffer, pH 7.5, with 0.02% Na₂S₂O₃ and 0.1% BSA in a final volume of 160 μ l. After 2 hr at 37°C, the precipitate was recovered by centrifugation (Microfuge, Beckman) through a discontinuous sucrose gradient composed of 50 μ l of 0.5 M sucrose and 100 μ l of 1.0 M sucrose in the phosphate-BSA buffer as described by Taylor and Schimke (20). The precipitate was analyzed on SDS-PAGE after measuring its radioactivity. Absorption experiments were carried out by incubating the antiserum with a great excess of nonlabeled protein for 30 min at 37°C, before adding it to the radiolabeled protein. Labeling of proteins with 125 I for these experiments was done in the same way as the radioiodination for RIA. In order to make high molecular impurities of α_1 -m and protein HC more prominent in the immunoprecipitation experiments, very early fractions were chosen from the Sephadex G-50 gel filtration that follows the labeling procedure to represent 125 I- α_1 -m and 125 I-protein HC.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Disc SDS-PAGE was done according to Neville (21). Separation gels had a total acrylamide concentration of 12% and the cross-linking was 3.3%. In some cases the gels were sliced into approximately 60 fractions, which were analyzed for 125 I in a gamma counter.

Determination of amino-terminal sequence. The amino-terminal sequence of α_1 -m was determined with the SDS-dansyl-Edman method outlined by Weiner *et al.* (22).

RESULTS

Quantitation of α_1 -m in solubilized cells. The solubilization treatment released no significant amounts of α_1 -m from any of the tested cells (Table I), when measured with RIA of α_1 -m with antisera 108 and 319; nor was the RIA of protein HC inhibited by the solubilized cells. The results did not differ from the data obtained when treating 0.25 M sucrose without cells in the manner outlined above. When the same treatment was given human serum or purified α_1 -m in bovine serum, full recovery was obtained. The detection limit, when measuring

TABLE I

Human cell types lacking α_1 -m and protein HC as measured with radioimmunoassay of cell culture media and in supernatants of solubilized cells

Cell Line ^a	Cell Type ^b	Cell Culture Medium		Supernatants of Solubilized Cells	
		α_1 -m	HC	α_1 -m	HC
Peripheral white blood cells ^c		—	—		
Normal blood lymphocytes ^c				—	—
Erythrocytes ^c				—	—
U-698	B lymphoma	—	—	—	—
Daudi	B lymphoma	—	—	—	—
U-255	Lymphoblastoid	—	—	—	—
U-974	Lymphoblastoid	—	—	—	—
U-296	Myeloma	—	—	—	—
RPMI 8226	Myeloma	—	—	—	—
MOLT ^c	T leukemia	—	—	—	—
CCRF-CEM	T leukemia	—	—	—	—
NALL-1	"Null" leukemia	—	—	—	—
K 562	Myeloid leukemia	—	—	—	—
U-143 S	Skin fibroblasts	—	—	—	—
U-1649 S	Skin fibroblasts	—	—	—	—
U-706	Glioma	—	—	—	—
Nj 310,320,330	Epithelial kidney cells	—	—	—	—

^a For references to the various cell lines, see Reference 23.
^b According to the classification proposed by Nilsson and Pontén (10).
^c Peripheral white blood cells, normal blood lymphocytes, and erythrocytes were taken from two healthy individuals.

released α_1 -m and protein HC from solubilized erythrocytes, was 5 to 30 molecules per cell and from the other cell types between 2000 and 8000 molecules per cell.

Quantitation of α_1 -m in cell cultures. α_1 -M or protein HC could not be detected in the culture media of any of the cell types as shown in Table I. Anti- α_1 -m sera 82, 108, and 319 were used. The peripheral white blood cells did not release any of the proteins after PHA-M was added to the medium. Smaller amounts of α_1 -m (and protein HC) than 0.03 to 0.10 ng/10⁵ cells for the established cell lines, and 0.10/2 × 10⁶ cells for the white blood cells, could not be detected. As a control experiment, all incubations were also analyzed for β_2 -microglobulin. The results of these determinations were always positive, and the amount of β_2 -microglobulin release ranged between 21 and 380 ng/10⁵ cells. The time-dependence of β_2 -microglobulin release in cultures of peripheral white blood cells, with or without PHA-M, agreed with that reported elsewhere (24), indicating a normal growth of these cells.

Immunofluorescence. Clearly positive reactions with anti- α_1 -m sera were only found against the normal peripheral lymphocytes. Three antisera were used (Table II), and each serum stained only a fraction of the peripheral lymphocytes. Some weak reactivity with antiserum 108 was found in a few additional lines. This reactivity was doubtful since in some instances a reaction with normal rabbit serum was simultaneously observed. In a limited number of tests (Table II) blocking experiments were performed by use of an excess of purified α_1 -m. In neither of the cell types tested was blocking successful. The reaction with anti-protein HC was frequently positive, and for a few cell types, shown in Table II, blocking tests were done. Protein HC could not inhibit the positive immunofluorescence in these experiments.

Antigenic relationships between α_1 -m and protein HC. On immunodiffusion analysis, α_1 -m and protein HC display a reaction of immunologic identity (Fig. 1). SDS-PAGE of Sephadex G-50-fractioned ¹²⁵I-labeled α_1 -m and protein HC reveals

TABLE II

Surface expression of α_1 -microglobulin in human in vitro cell lines

Cell Line ^a	Cell Type ^b	Indirect Immunofluorescence Serum				
		82	83	108	HC	NRS ^c
U-255	Lymphoblastoid			(+)	+	—
U-974	Lymphoblastoid			—	+	—
U-VO	Lymphoblastoid			—	++	—
U-698	B lymphoma	—	—	(+)	+	—
U-715	B lymphoma	—			—	—
DG-75	B lymphoma	—			+	—
Daudi	B lymphoma	—	—	—	—	—
Raji	B lymphoma			—	—	—
DHL-4	B lymphoma			—	+	—
Platz	B lymphoma			(+)	+	—
U-937	Histiocytic lymphoma	—	—	(+)	+++	—
U-266	Myeloma	—	—	—	+	— ^d
RPMI 8226	Myeloma			—	+	(+)
MOLT	T leukemia	—	—	—	—	—
CCRF- HSB2	T leukemia	—	—	—	—	—
CCRF-CEM	T leukemia	—	—	—	—	—
JM	T leukemia	—	—	(+)	—	—
NALL-1	"Null" leukemia			(+)	++	—
KM 3	"Null" leukemia			(+)	—	(+)
BALL	B leukemia			—	+	—
K 562	Myeloid leukemia	—	—	—	—	—
U-143 S	Skin fibroblasts	—	—	—	—	—
U-118 Mg	Glioma	—	—	—	—	—
U-251 Mg	Glioma	—	—	—	—	—
U-706	Glioma	—	—	—	—	—
Normal lymphocytes		+	+	+	+++	— ^d
CLL 1	Chronic lymphocytic leukemia	(+)		—	+++	—
U-1285	Anaplastic carcinoma			(+)	+++	— ^d

^a For references to the various cell lines, see Reference 23.
^b According to the classification proposed by Nilsson and Pontén (10).
^c Normal rabbit serum.
^d Blocking experiments performed.

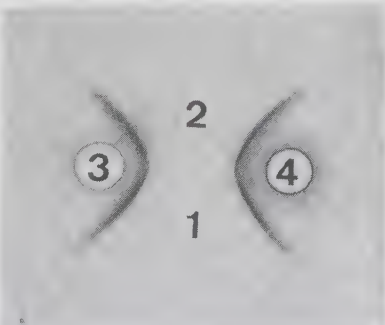


Figure 1. Ouchterlony immunodiffusion showing immunologic identity between α_1 -m (1) and protein HC (2), using anti- α_1 -m 108 (3) and anti-protein HC (4).

two additional peaks beside the major component, with an apparent m.w. of 31,000 d (Fig. 2a, d). Both preparations reveal a component with the same mobility as albumin. In the protein HC sample, there is also a component of approximately 50,000 d, and a contamination of higher m.w. than albumin appears to be present in the α_1 -m preparation. There are small differences in the way anti- α_1 -m and anti-protein HC reacts with the two antigens and their contaminations. Both antisera precipitate a smaller portion of the high m.w. components than of the main 31,000 d peak (Fig. 2b, c, e, f). No significant difference between the two proteins or their antisera could be seen in the inhibition experiments of immunoprecipitation. Anti- α_1 -m serum was blocked by protein HC with approximately the same efficiency as by α_1 -m when binding radiolabeled α_1 -m or protein HC. The same was the case with anti-protein HC. The slopes of the RIA-inhibition curves of these four systems were parallel when using either α_1 -m or protein HC as the inhibiting antigen.

Anti- α_1 -acid glycoprotein could bind significant amounts of radioactivity from 125 I-labeled α_1 -m and protein HC. The immunoprecipitates were subjected to SDS-PAGE and the results are shown in Figure 3. A single, well defined peak of approximately 45 to 50,000 d was bound from protein HC, whereas the precipitated material from α_1 -m migrated as multiple peaks in the 30 to 80,000 d region.

Absorption of anti- α_1 -m serum with cells. Figure 4 shows the differences between absorbed and unabsorbed anti- α_1 -m serum 108 reacting with 125 I- α_1 -m (Fig. 4a, c) or 125 I-protein HC (Fig. 4b, d). Peripheral white blood cells bound antibodies with affinity to the 50,000 d component. This peak was reduced when precipitating the α_1 -m or protein HC preparations with

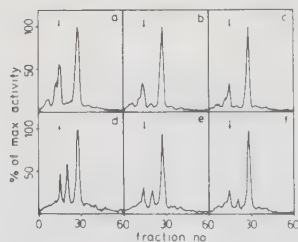


Figure 2. SDS-PAGE of Sephadex G-50 fractionated 125 I- α_1 -m (a to c) and 125 I-protein HC (d to f), precipitated by anti- α_1 -m serum 108 (b, e), anti-protein HC serum (c, f) and not precipitated (a, d). The arrows indicate the position of human albumin.

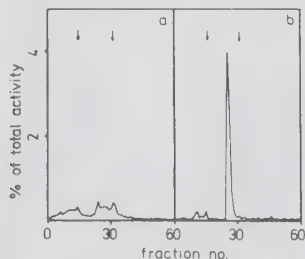


Figure 3. SDS-PAGE of Sephadex G-50 fractionated 125 I- α_1 -m (a) and 125 I-protein HC (b), precipitated by anti- α_1 -acid glycoprotein serum. The units on the y-axis represent radioactivity as per cent of total activity in the 125 I- α_1 -m or 125 I-protein HC sample. The arrows indicate the position of human albumin and α_1 -m.

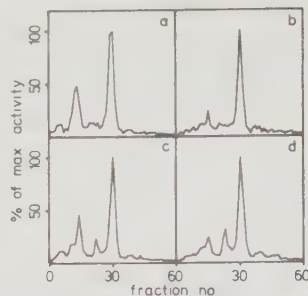


Figure 4. SDS-PAGE of Sephadex G-50 fractionated 125 I- α_1 -m (a, c) and 125 I-protein HC (b, d), precipitated by anti- α_1 -m serum 108 absorbed with normal leukocytes (a, b) and not absorbed (c, d).

α_1 -Microglobulin: Gly - Pro - Val - Pro - Thr - Pro - Pro
Protein HC: Gly - Pro - Val - Pro - Arg - Pro - Pro

Figure 5. Amino-terminal sequence of α_1 -m compared to the corresponding residues of protein HC (25, 26).

the absorbed antiserum. Similar results were obtained when using the lymphoid cell line NALL-1.

Amino-terminal sequence of α_1 -m. The first seven amino acids of the amino-terminus of α_1 -m are shown in Figure 5. They are compared to the corresponding sequence of protein HC. Identical results were obtained, except for the fifth residue. This residue of protein HC was reported to be arginine (26), whereas it was identified as threonine in α_1 -m.

DISCUSSION

We have not been able to detect any release of α_1 -m from peripheral lymphocytes, erythrocytes, or two lymphoid cell lines when treating these with various solubilization techniques. There is the possibility that the solubilization treatment affects the antigenicity of α_1 -m, making it undetectable in the RIA. However, no such effect could be seen on purified α_1 -m, or α_1 -m in bovine serum. There seems to be no *in vitro* production of α_1 -m by peripheral lymphocytes or lymphoid cell lines, as indicated by the failure of these cell culture media to inhibit anti- α_1 -m in a RIA. We were not able to detect less than 0.1 ng/ml of α_1 -m in the supernatants. The release of β_2 -microglobulin from these cell lines indicates that the missing α_1 -m was not likely to be due to an impaired cell growth. Immunofluorescence experiments with anti- α_1 -m failed to stain most lymphoid and nonlymphoid cell lines, and only normal lymphocytes were stained weakly. The positive immunofluorescence could not be inhibited by purified α_1 -m when tested, suggesting that the immunofluorescence on lymphocytes is not specific for α_1 -m.

Protein HC has been shown to be expressed on a number of different cell types by means of immunofluorescence (4, 5). Peripheral lymphocytes, erythrocytes, and fibroblast cell lines showed strong membrane fluorescence. Protein HC is by a number of physicochemical criteria identical to α_1 -m (2, 3, 25). In this study we have shown that the amino-terminal sequence of α_1 -m is in agreement with that of protein HC, except for the fifth residue (25, 26). The antigenic behavior of α_1 -m and protein HC seems to be similar. The gel-diffusion and immunoprecipitation experiments demonstrate immunologic identity between the two proteins. Any difference between α_1 -m and protein HC and their antisera must therefore be due to minor components,

i.e., impurities in the preparations of the proteins. As we have shown in experiments accentuating high m.w. contaminations, there are impurities in the α_1 -m and protein HC preparations. We have been able to precipitate material with anti- α_1 -acid glycoprotein serum (Fig. 3). Gahmberg and Andersson (27) have recently shown α_1 -acid glycoprotein to be present on the surface of leukocytes. Antibodies in the anti- α_1 -m or anti-protein HC sera against α_1 -acid glycoprotein could very well react with the orosomucoid known to be present on the surface of leukocytes.

In the absorption experiments with anti- α_1 -m, we have analyzed the specificity of the antibodies that bind to normal leukocytes and the cell line NALI-1. They are directed against the 50,000 d component as shown in Figure 4, not against α_1 -m or protein HC.

As a competitive immunologic method, the RIA of α_1 -m is relatively insensitive to minor components in the mixture of antigenically active molecules. The immunofluorescence technique, on the other hand, can not discriminate between minor and major components. Consequently, the negative data with RIA and the positive immunofluorescence on certain cell membranes could be explained if one assumes that one or more of the impurities, not α_1 -m or protein HC, is responsible for the membrane fluorescence, especially since it could not be blocked by the purified proteins. The fact that different anti- α_1 -m sera stain certain cell membranes with different intensity—in some cases one antiserum is negative where another antiserum is positive—adds to the probability of this explanation.

The detection limit of α_1 -m release in our experiments with leukocytes is 0.1 ng per 2×10^6 cells and 3 days. If all of the approximately 2×10^{12} leukocytes in a human body (H. Wigzell, personal communication) would uniformly release α_1 -m at this rate, 0.1 mg, or 0.4% of the 27 mg of α_1 -m excreted in the urine (2), would be supplied by the leukocytes. Since the urinary excretion of a low m.w. plasma protein usually is very small compared to the amount eliminated from serum by reabsorption in tubuli, for λ -L-chain and lysozyme $\sim 1\%$ (28), the rate of synthesis of α_1 -m has to be considerably greater than the urinary excretion. These calculations suggest that it is highly improbable that the lymphocytes are a major source of α_1 -m.

Tejler *et al.* (29) have demonstrated the synthesis of α_1 -m from human fetal liver explants. Unpublished observations (B.Å. and I.B.) suggest that cultured rat-liver cells release the rat homologue of α_1 -m. These data, and the results presented in this paper, indicate that cells other than of lymphoid origin are responsible for the production of α_1 -m.

Acknowledgments. Protein HC and anti-protein HC were given to us by Dr. Anders Grubb. Anti- α_1 -acid glycoprotein was a gift from Dr. Leif C. Andersson, Helsinki, Finland. We also wish to thank Dr. Per A. Peterson for valuable help and discussions.

REFERENCES

- Ekström, B., P. A. Peterson, and I. Berggård. 1975. A urinary and plasma α_1 -glycoprotein of low molecular weight: isolation and some properties. *Biochim. Biophys. Res. Commun.* 65:1427.
- Ekström, B., and I. Berggård. 1977. Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J. Biol. Chem.* 252:8048.
- Tejler, L., and A. O. Grubb. 1976. A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine and cerebrospinal fluid. *Biochim. Biophys. Acta* 439:82.
- Tejler, L., A. O. Grubb, and I. Tureson. 1976. Immunofluorescent demonstration of the presence of protein HC on the surface of lymphocytes. *Acta Med. Scand.* 199:425.
- Pearlstein, E., I. Tureson, L. Tejler, and A. O. Grubb. 1977. Expression of protein HC on the plasma membrane of different human cell types. *J. Immunol.* 119:824.
- Boyum, A. 1968. Separation of leukocytes from blood and bone-marrow. *Scand. J. Clin. Lab. Invest.* 21 (Suppl. 97):9.
- Kissmeyer-Nielsen, F., and K. E. Kjerbye. 1967. Lymphocytotoxic microtechnique. Purification of lymphocytes by flotation. *In* Histocompatibility Testing. Munksgaard. Copenhagen. P. 381.
- Hedberg, H., B. Källén, B. Löw, and O. Nilsson. 1971. Impaired mixed leukocyte reaction in some different diseases, notably multiple sclerosis and various arthritides. *Clin. Exp. Immunol.* 9:201.
- Berggård, I. 1961. Proteins, glycoproteins and mucopolysaccharides in normal human urine. *Ark. Kemi* 18:291.
- Nilsson, K., and J. Pontén. 1975. Classification and biological nature of established human hematopoietic cell lines. *Int. J. Cancer* 15:321.
- Berggård, I., and A. G. Bearn. 1968. Isolation and properties of a low molecular weight β_2 -globulin occurring in human biological fluids. *J. Biol. Chem.* 243:4095.
- Björck, L., R. Cigén, B. Berggård, B. Löw, and I. Berggård. 1977. Relationships between β_2 -microglobulin and alloantigens coded for by the major histocompatibility complexes of the rabbit and the guinea pig. *Scand. J. Immunol.* 6:1063.
- Evvin, P. E., and H. Perotto. 1973. β_2 -Microglobulin in human blood cells. *J. Immunol.* 111:1147.
- Nilsson, K., and C. Sundström. 1974. Establishment and characteristics of two unique cell lines from patients with lymphosarcoma. *Int. J. Cancer* 14:808.
- Sundström, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* 16:565.
- Plesner, T., B. Nørgaard-Pedersen, and T. Boenisch. 1975. Radioimmunoassay of β_2 -microglobulin. *Scand. J. Clin. Lab. Invest.* 35:729.
- Greenwood, F. C., and W. M. Hunter. 1963. The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114.
- Ouchterlony, O. 1958. Diffusion-in-gel methods for immunological analysis. *Progr. Allergy* 5:1.
- Berggård, I. 1961. Studies of the plasma proteins in normal human urine. *Clin. Chim. Acta* 6:413.
- Taylor, J. M., and R. T. Schimke. 1973. Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. *J. Biol. Chem.* 248:7661.
- Neville, D. M., Jr. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* 246:6328.
- Weiner, A. M., T. Platt, and K. Weber. 1972. Amino-terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis. *J. Biol. Chem.* 247:3242.
- Nilsson, K. 1979. The nature of lymphoid cell lines and their relationship to EB virus. *In* The Epstein-Barr Virus. Edited by F. A. Epstein and B. G. Achong. Springer Verlag, Heidelberg.
- Bernier, G. M., and M. W. Fanger. 1972. Synthesis of β_2 -microglobulin by stimulated lymphocytes. *J. Immunol.* 109:407.
- Frangione, B., E. C. Franklin, A. Grubb, and L. Tejler. 1976. The amino-terminal sequence of human protein HC. *F.E.B.S. Lett.* 70:239.
- Tejler, L. 1978. Ph.D. Thesis. University of Lund, Sweden.
- Gahmberg, C. G., and L. C. Andersson. 1978. Leukocyte surface origin of human α_1 -acid glycoprotein (orosomucoid). *J. Exp. Med.* 148:507.
- Strober, W., and T. A. Waldmann. 1974. The role of the kidney in the metabolism of plasma proteins. *Nephron* 13:35.
- Tejler, L., S. Eriksson, A. Grubb, and B. Åstedt. 1978. Production of protein HC by human fetal liver explants. *Biochim. Biophys. Acta* 542:506.

Immunosuppressive Properties of α_1 -Microglobulin

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α_1 -Microglobulin (α_1 m), a serum glycoprotein (26,000 d), was found to impede the proliferative response of human lymphocytes to purified protein derivative (PPD) and tetanus toxoid. The data suggest that α_1 m operates through an unstable suppressor mechanism, which no longer can function after 24 h of preculturing. This effect of α_1 m on antigen stimulation did not seem to be due to binding of α_1 m to PPD or cells, to altered kinetics of the PPD response, or to non-specific cytotoxicity. In contrast, PPD-induced leucocyte migration inhibition was not reversed by α_1 m. α_1 m did not cause significant inhibition in experiments in which lymphocytes were stimulated by the mitogens phytohaemagglutinin or concanavalin A. Finally, α_1 m had its own leucocyte migration inhibitory effect.

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Recently, a low molecular weight plasma protein, α_1 -microglobulin (α_1 m), was isolated from the urine of patients with disorders in the proximal tubules of the kidney [10]. It was described as a glycoprotein with a molecular weight of about 26,000, containing approximately 20% carbohydrate. α_1 m is electrophoretically heterogeneous, carries a brown-coloured substance, and is present in serum both in free form and in high molecular weight complexes [9, 10]. Four other groups have since reported data on α_1 m [18–21].

The function and site of synthesis of α_1 m is still unknown. Several reports have implicated immunoregulatory properties within the α -globulin region of the plasma proteins (cf. Ref. 22). We have now investigated the effect of α_1 m on two in vitro cell-mediated immune reactions, antigen-induced lymphocyte proliferation and leucocyte migration inhibition.

MATERIALS AND METHODS

α_1 m. α_1 m was purified from the urine of patients with renal damage. Two different purification schedules were used. First, α_1 m was isolated from three different indi-

viduals as described earlier [9]. These three preparations will be referred to in the text as α_1 m preparations I, II and III. Second, α_1 m was isolated along an alternate purification scheme adopted to provide a gentler and faster urine sample handling and a higher resolution between α_1 m and the high molecular weight contaminants. A fresh 24-h urine specimen was dialysed against 0.05 M Tris-HCl, pH 7.7, + 1.0 M NaCl. After the dialysis, $MgCl_2$, $CaCl_2$ and $MnCl_2$ were added to 1 mM each, and the urine was subjected to a concanavalin A (Con A)-Sephadex column (15 $\times$ 6 cm) equilibrated with 0.05 M Tris-HCl, pH 7.7, containing 1 M NaCl and 1 mM $MgCl_2$, $CaCl_2$ and $MnCl_2$. The column was thoroughly washed with this buffer, and then eluted with 0.1 M α -methyl-D-glucoside in the same buffer. The protein thus eluted was concentrated and gel chromatographed on a Sephadex G-200 column (140 $\times$ 5 cm). The further purification of the α_1 m by preparative zone electrophoresis, gel chromatography on Sephadex G-100, and ion-exchange chromatography on DEAE-Sephadex A-50 was identical to the original isolation procedure [9]. In this way α_1 m was isolated from two individuals and from pooled urine, resulting in three preparations, referred to in the text as α_1 m preparations IV, V and VI, respectively. Although highly purified, α_1 m prepared as originally described [9] contained minor impurities, as discussed elsewhere [2]. It can be shown with radioimmunological techniques that the α_1 m purified as described above was even more highly purified.

In one experiment, separating the α_1 m dimer from the monomer, 40 mg of the purified protein were applied to a Sephadex G-100 column (100 $\times$ 3.5 cm),

equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% Na₂S₂O₃. 1.5 ml of fifteen representative fractions were dialysed against distilled, deionized water, freeze-dried, and dissolved in 0.2 ml phosphate-buffered saline (PBS) before they were added (10 µl) to separate incubation tubes for the leucocyte migration experiments.

Other proteins. Purified protein derivative (PPD) was purchased from Statens Seruminstitut, Copenhagen, Denmark, and used at 50 µg/ml (optimum dose), unless otherwise stated. Tetanus toxoid was from The National Bacteriological Laboratory (SBL), Stockholm, Sweden. It was used at a protein concentration of 5 µg/ml, corresponding to 2.5 Lf/ml. Phytohaemagglutinin-M (PHA) and Con A were from Sigma, St Louis, Mo., USA. After reconstitution, PHA was used at three concentrations, one supra-optimum (100 µl/ml), one optimum (5 µl/ml), and one suboptimum (0.5 µl/ml), and Con A was used at 50 µg/ml. Bovine serum albumin (BSA) was from Sigma, and highly purified orosomucoid [11] was kindly provided by Dr Leif C. Andersson, Helsinki, Finland. BSA and orosomucoid were used at 1 mg/ml.

Cells. Blood was drawn from seemingly healthy human donors and peripheral leucocytes were separated from erythrocytes by sedimentation in dextran (Macrodex, Pharmacia, Uppsala, Sweden) [13].

Cell cultures and assay of DNA synthesis. Cell culturing was performed essentially as previously detailed [13]. Each culture consisted of 1×10^6 leucocytes in 0.5 ml medium (20% (v/v) human serum in Parker 199 (SBL, Stockholm) with antibiotics added) and additions of PPD, tetanus toxoid, PHA, Con A, saline, α_1 m (25–50 µl), or combinations of these. The cultures were kept and in air atmosphere. Triplicates of parallel cultures were always performed. The culture time was 3 days for PHA and Con A stimulations (in one experiment 7 days) and 7 days for PPD and tetanus toxoid stimulations. At the end of the culture period, tritiated thymidine (methyl-³H-thymidine, Schwartz/Mann, Orangeburg, USA; 1.9 Ci/mm, final concentration 2 µCi/ml) was added and the culture continued for another hour. The cells were then harvested and subjected to liquid scintillation counting with a scintillation system consisting of 0.5 ml Soluene 350 and 2.5 ml Insta-Fluor (both Packard, Downers Grove, USA). Culturing, harvesting, and liquid scintillation were performed in the same disposable plastic tubes (GBR Instruments, Hvidovre, Denmark).

Leucocyte migration. Leucocyte migration under agarose was performed as described [4, 6] with slight modifications. Briefly, peripheral blood leucocytes, 220×10^6 /ml, were distributed in aliquots of 50 µl (serum-free Parker 199 medium). Some aliquots contained additions of PPD, α_1 m, BSA, orosomucoid, or PPD together with α_1 m (5–10 µl). After incubation at 37°C for 30 min, the cells were distributed in holes (7 µl) punched in agarose gels (containing 10% normal horse serum (Flow Laboratories, Ayrshire, Scotland)) in 5-cm plastic Petri dishes. Each incubation was tested on six parallel gels. A gel could hold a maximum of fifteen holes. Thus, up to fifteen incubations could be tested in one experiment. The plates were incubated in 5% CO₂ in air (v/v) at 37°C for 24 h, after which they were fixed with methanol and the gels were removed.

The migration areas were then determined and expressed in units corresponding to 0.08 mm².

Other methods. Ouchterlony double diffusion [16] was performed as described [3].

Radiolabelled [12] α_1 m, incubated in 1 ml culture medium for 24 h at 37°C, with or without 100 µg PPD, was chromatographed on a Sephadex G-200 column. The column (90 × 1.6 cm) was equilibrated and eluted with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% Na₂S₂O₃, and the flow rate was 2.2 ml/h.

α_1 m (10 ng/ml) labelled with ¹²⁵I, and PPD (50 µg/ml) were incubated with 1×10^7 peripheral blood leucocytes in 5 ml Parker 199 for 4 h at room temperature. The incubations were performed with or without the presence of 'cold' α_1 m (100 µg/ml). The cells were washed thoroughly with Parker 199 at 4°C before the radioactivity of the cells was counted. The cells had been pretreated in PBS for 6 h at room temperature.

Statistical considerations. The results of lymphocyte activation experiments, received as counts per 10 min, were transformed to log₁₀cpm values to create a near-normal distribution of the variates [13]. Log₁₀ of stimulation indices (log₁₀SI = mean log₁₀cpm of stimulated cultures minus mean log₁₀cpm of control cultures) were used to evaluate effects of PPD, tetanus toxoid, Con A, and PHA. The inhibitory activity of α_1 m on these effects was evaluated as log₁₀SI (for PPD, tetanus toxoid, Con A, or PHA) minus log₁₀SI in the presence of α_1 m. The degree of inhibition, caused by α_1 m, was also examined as percentage residual activity: $100 \times (\text{SI (with } \alpha_1\text{m)} - 1) / (\text{SI} - 1)$. *t* tests were performed to ascertain the significance of effects and inhibitory activity on effects. The tests were based on the technical error as estimated from the variation within 'identical' triplets (SD = 0.191, 684 df, 7 days; SD = 0.127, 64 df, 3 days).

Migration indices (MI) calculated as (test migration/control migration) × 100, were used to evaluate the leucocyte migration experiments. Migration inhibition is expressed in per cent (100 – MI). *t* tests based on the technical error, as estimated from the variation within groups of six 'identically' prepared migrations, showed that about 10% migration inhibition was necessary to reach statistical significance.

RESULTS

Effect of α_1 m on lymphocyte activation induced by soluble antigens

Table I presents data from PPD stimulations of cells from nine subjects. The effects of α_1 m preparations I and II (0.3 mg/ml) on each of these were tested in one experiment per individual. α_1 m inhibited the PPD stimulations, in most cases to a statistically significant extent. The two α_1 m preparations (I and II) appeared in most cases similar in their inhibitory capacity, but for two persons (2 and 4), their inhibitions differed significantly. The effect of α_1 m on background thymidine uptake was in general

TABLE I. Inhibitory effect of α_1 -microglobulin on PPD-induced lymphocyte transformation†

Subject	PPD effect, $\log_{10}SI$	Inhibitory activity of α_1m on PPD effect	
		Preparation I (% residual activity)	Preparation II (% residual activity)
1	1.69	0.53* (28)	0.93*** (10)
2	0.57	0.55* (2)	0.09NS (74)
3	1.70	0.34NS (45)	0.55* (27)
4	1.42	0.06NS (87)	0.56* (25)
5	1.25	-0.22NS (170)	-0.27NS (191)
6	0.73	0.06NS (84)	0.06NS (84)
7	1.66	1.08*** (6)	0.83*** (13)
8	1.27	0.91*** (7)	0.81*** (11)
9	1.65	0.44* (35)	0.55* (27)
Mean $\pm$ SEM	1.33 ± 0.14	0.42 ± 0.14 (35)	0.45 ± 0.14 (32)

† α_1m was used at 0.3 mg/ml. NS -- not significant, * $0.05 > P > 0.01$; *** $0.001 > P$.

negligible: 0.04 ± 0.04 and -0.11 ± 0.03 for preparations I and II, respectively (mean $\pm$ SEM, 8 df). No visible precipitates were formed between α_1m and PPD, at various concentrations, on Ouchterlony double diffusion plates. Addition of PPD did not alter the elution volume, on a column of Sephadex G-200, of radiolabelled α_1m in culture medium.

α_1m preparation III (0.3 mg/ml) inhibited the lymphocyte transformation induced by a second

TABLE II. Inhibitory effect of α_1 -microglobulin on tetanus toxoid-induced lymphocyte transformation†

Subject	Tetanus toxoid effect, $\log_{10}SI$	Inhibitory activity of α_1m on tetanus toxoid effect (% residual activity)
1	0.99	0.78*** (7)
2	1.37	1.11*** (4)
3	1.22	0.22 NS (58)
4	1.09	0.51* (25)

* $0.05 > P > 0.01$; *** $0.001 > P$.

† α_1m preparation III was used at 0.3 mg/ml.

NS = not significant.

soluble antigen, tetanus toxoid (see Table II). Preparations IV and V of α_1m (0.3 mg/ml) also inhibited the lymphocyte stimulations induced by the two soluble antigens.

Some characteristics of the α_1m inhibitory effect on the PPD stimulation of lymphocytes

Fig. 1 shows, in a representative experiment, the effects of different doses of α_1m on the PPD stimulation of lymphocytes. With increasing doses of α_1m the PPD response gradually diminished. At 0.3 mg α_1m /ml, this inhibition was statistically significant. At 1 mg/ml, α_1m impaired the background thymidine uptake significantly. The different lymphocyte responses to PPD at four different concentrations (1, 3, 12 and 50 μ g/ml) were inhibited to the same extent (residual activity in one experiment: 25%, 43%, 45% and 35%, respectively) by α_1m at 0.3 mg/ml.

The inhibitory effect of α_1m on PPD stimulation of lymphocytes (Fig. 2a-d) was not seen when the cells were preincubated for 24 h and washed before initiation of the tests (Fig. 2e-h). The thymidine uptake of the PPD cultures with α_1m (Fig. 2h) was now the same as without α_1m (Fig. 2f). Instead, the background thymidine uptake (Fig. 2e) was often significantly increased by the addition of α_1m (Fig. 2g). These pre-incubation experiments were performed separately with cells from two subjects, and with similar results.

Effect of α_1m on lymphocyte activation induced by mitogens

In repeated experiments, α_1m (0.3 mg/ml)

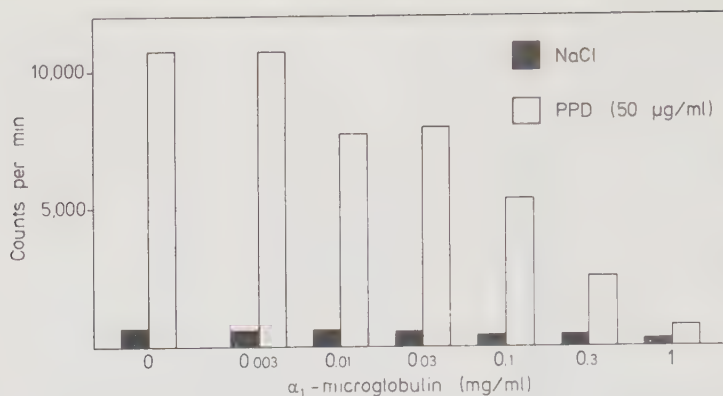


FIG. 1. Effect of various concentrations of α_1 -microglobulin (preparation III) on PPD-stimulated and on control (NaCl) cultures. The bars represent the ^3H -thymidine uptake expressed as geometric means of triplicate cultures.

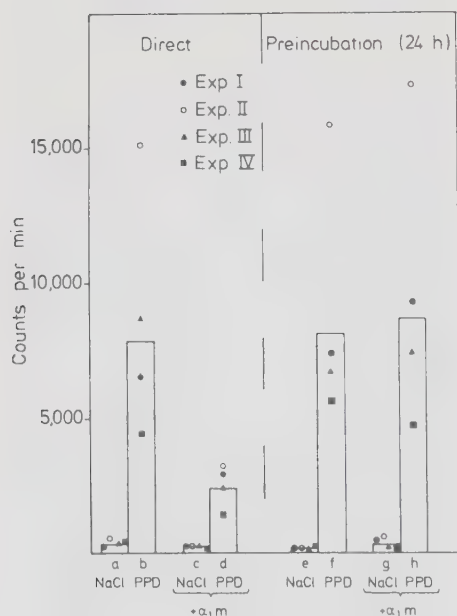


FIG. 2. Effect of α_1 -microglobulin (preparation II) on PPD stimulation of fresh (a-d) or precultured (e-h) cells. The figure shows four different experiments with cells from the same subject. Each dot represents the geometric mean of triplicate PPD-stimulated or control (NaCl) cultures. The bars show geometric means of the four different experiments. The precultured cells were washed before addition of reagents, after which they were cultured for 7 more days. α_1 m was used at 0.3 mg/ml.

failed to elicit significant inhibition of PHA stimulation with various PHA concentrations. When PHA-induced lymphocyte stimulation was prolonged to 7 days, α_1 m still could not significantly block the response. Similar results were obtained with Con A.

Effect of α_1 m on PPD-induced leucocyte migration inhibition

The effect of α_1 m (0.3 mg/ml) on PPD-induced leucocyte migration inhibition was tested on ten subjects (see Fig. 3). In no case did α_1 m significantly reverse the migration inhibitions caused by PPD. Rather, the migration inhibitions were slightly increased by the addition of α_1 m.

The α_1 m-induced leucocyte migration inhibitory effect

α_1 m alone could inhibit leucocyte migration (Fig. 3). All six α_1 m preparations were tested and found to have an inhibitory effect on leucocyte migration. Fig. 4 shows the effects of various concentrations of α_1 m on leucocyte migration of cells from four different individuals. In general, the leucocyte migration diminished as the α_1 m concentration increases. No obvious plateaus were seen in this dose interval.

Fig. 5, top, shows the distribution of purified

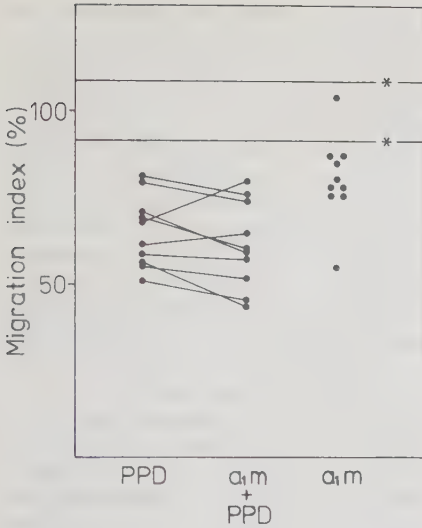


FIG. 3. Effect of α_1 -microglobulin (preparation II) on PPD-induced (50 μ g/ml) leucocyte migration inhibition. The results are expressed as percentages of control migration, each dot representing the mean of six migrations. Ten different subjects were tested. Dots representing the same persons are combined with lines. α_1m was used at 0.3 mg/ml. Statistical significance of deviation from control migration, at the 5% level (*), is indicated. The effect of α_1m alone is also shown.

α_1m separated on a Sephadex G-100 column. It is eluted at two positions, probably corresponding to dimers and monomers. The distribution of migration inhibitory capacity as

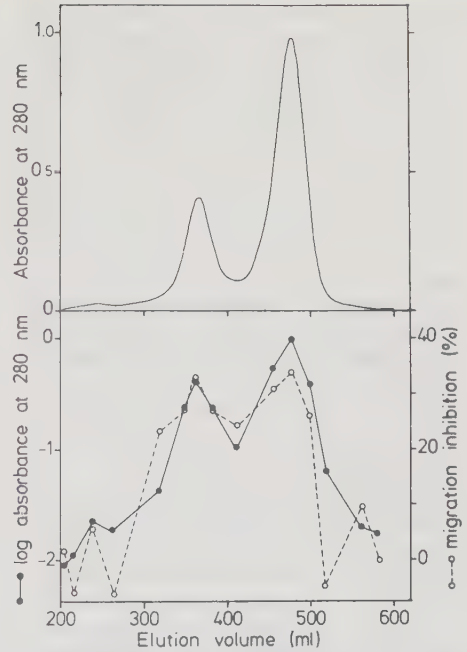


FIG. 5. Sephadex G-100 gel chromatography of purified α_1m preparation VI (top). The inhibitory activity in leucocyte migration of fifteen different fractions is compared with the logarithm of the absorbance of the same fractions (bottom). The fractions were tested for migration inhibitory activity on three subjects (mean values) at a concentration of 1.25 times the concentrations in the eluted fractions. The absorbance of 1 mg α_1m /ml is 1.82.

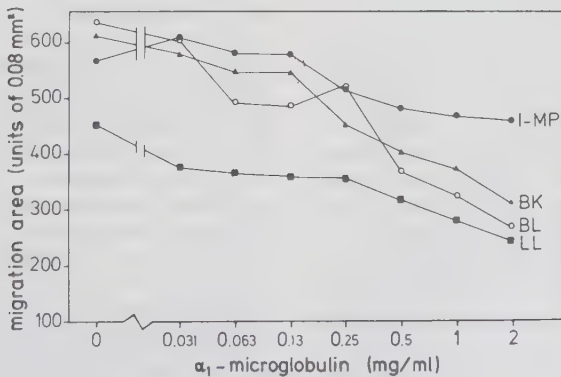


FIG. 4. Effects of various concentrations of α_1 -microglobulin (preparation III) on leucocyte migration. Cells from four different individuals were tested. Direct migration areas are given and represent means of six simultaneous migrations.

measured in three subjects coincides with these peaks (Fig. 5, bottom). The similarity between the two plots is accentuated if protein distribution is plotted on a log scale (Fig. 5, bottom).

BSA or orosomucoid (1 mg/ml) did not inhibit leucocyte migration.

Incubation of radiolabelled α_1m with peripheral blood leucocytes

Radiolabelled α_1m did not bind to peripheral blood leucocytes (<100 molecules/cell or, for instance, 10^4 molecules on 1% of the cells), when ^{125}I - α_1m , PPD, and the cells were incubated for 4 h at room temperature.

DISCUSSION

The present work demonstrates that α_1m can inhibit antigen-induced lymphocyte activation by two soluble antigens. The fact that α_1m did not affect the background thymidine uptake indicates that the α_1m -induced inhibitory effect was specific for the antigen stimulations and not due to non-specific cytotoxicity. A physical interaction between α_1m and the soluble antigens could be a cause of the inhibitory effects of α_1m , even though it is improbable that α_1m would react with both PPD and tetanus toxoid. Evidence against such an interaction was obtained. PPD and α_1m did not form precipitates in Ouchterlony double-diffusion experiments, nor could preincubation with PPD change the elution volume of radiolabelled α_1m in culture medium on gel chromatography on a Sephadex G-200 column.

The dose-response relationship (Fig. 1) of the α_1m -induced inhibitions of PPD stimulations made us select 0.3 mg α_1m /ml as a standard concentration in most of the tests. Lower concentrations did not yield statistically significant inhibitions, and with the higher dose (1 mg/ml) the background thymidine uptake was significantly decreased. The selected concentration (0.3 mg/ml) is approximately 3–10 times that present in normal plasma [9]. The lymphocyte stimulation of suboptimum doses of PPD was inhibited by α_1m to the same extent as an optimum response. Therefore, only the optimum PPD dose (50 μ g/ml) was used in the other experiments. Time studies (not shown) indicated that the inhibitory activity of α_1m was not a

result of altered kinetics, such as an earlier peak response.

To investigate the stability of the suppressor mechanism by which α_1m inhibits the antigen stimulations, cells were preincubated for 24 h (Fig. 2). The inhibitory activity of α_1m now seemed reversed, assuming that the increased background thymidine uptake of the pre-cultured cells, caused by α_1m , is additive to the PPD effect. This reasonable assumption allows a clear interpretation of the results. Thus, the data suggest that α_1m acts through an unstable suppressor mechanism that is inactivated during the 24 h of preculture.

It has previously been shown that α_1m is probably not a lymphocyte membrane protein [2]. We have demonstrated in this work that radiolabelled α_1m , incubated with extensively washed peripheral blood leucocytes and PPD, does not bind to the cells. Thus, we have been unable to demonstrate that α_1m , when inhibiting the lymphocyte response to antigens, acts through direct binding to a cellular receptor for the protein. The detection limit of this experiment does not, however, permit us to exclude a binding of α_1m to a minor cell fraction (e.g. 10^4 molecules on 1% of the leucocytes).

Different traits of cell-mediated immunity (e.g. lymphocyte transformation and inhibition of leucocyte migration) could be expected to share a common induction phase. If so, α_1m apparently does not interact with this early phase, since PPD-induced inhibition of leucocyte migration was not reversed. Failure to inhibit mitogen stimulation with α_1m concentrations that depress antigen stimulation indicates a further selectivity of the suppressor mechanism involved.

α_1m has its own leucocyte migration inhibitory effect. This could be another interaction of α_1m with the immune system. For instance, α_1m may stimulate lymphocytes to produce leucocyte migration inhibitory factor [17] or may inhibit their spontaneous production of leucocyte migration enhancement factor [24]. Another possibility is that α_1m has chemotactic properties.

The inhibitory effects of α_1m on leucocyte migration and antigen-induced lymphocyte activation have been established with all α_1m preparations examined, i.e. preparations I–VI and I–V, respectively. However, magnitudal differences in the inhibitory activity of different

preparations were occasionally seen (cf. Table I). α_1m is electrophoretically heterogeneous and can be separated into at least six forms with different isoelectric points [1]. It is conceivable that the suppressive activities differ among these forms. The various α_1m preparations show quantitative differences in subgroup composition. This might be a reason for the discrepancies in inhibitory activities among α_1m preparations.

Another possibility is that the inhibitory effects are due to contaminants and not to α_1m itself. Although highly purified [9], we can detect minute amounts of orosomucoid in α_1m prepared according to the original scheme [2]. Interactions of orosomucoid with the immune system have been reported [5]. In the lymphocyte activation experiments, however, the contribution made by contaminating orosomucoid is negligible compared with the amounts of this protein in the medium-supporting serum. Moreover, the contaminating orosomucoid has been largely removed with the modified purification schedule. Thus, it is not probable that orosomucoid is responsible for the described inhibitions of antigen stimulation. Similarly, it is improbable that possible minor contaminations with other serum proteins, claimed to be immunosuppressive (e.g. Refs. 7, 8, 14 and 15), can explain our results.

The leucocyte migration inhibition caused by α_1m is not simply an effect of high protein concentrations. BSA and orosomucoid in similar concentrations were not inhibitory. The self-association property of α_1m [9] was used to examine the possibility that the inhibitory effect could be caused by contaminants. Thus, purified monomeric α_1m was separated on a Sephadex G-100 column, where it was eluted as two peaks, representing monomers and probably dimers (Fig. 5). If the inhibitory component is a contaminant, the migration inhibitory activity would not be expected in the newly formed peak. However, the migration inhibitory activity coincided with the protein peaks. The similarity of the two distributions was especially evident if the protein distribution was plotted on a log scale. Such a plot is suggested by the shape of the dose-response curves (Fig. 4). Thus, it seems likely that α_1m itself causes the leucocyte migration inhibitions.

To conclude, we have demonstrated a suppressive effect of α_1m on antigen-induced

lymphocyte stimulation. α_1m appears to mediate this inhibitory effect by activation of an unstable suppressor mechanism, which is operable only during the first 24 h of culture. This effect of α_1m , as well as its migration inhibitory effect, may be mediated by only a few specific subpopulations of α_1m . Likewise, the activity of α_1m could reside in the carbohydrate, peptide, or chromophore materials, or in combinations of these.

α_1m , at normal plasma levels, does not exert immunosuppressive effects detectable in our assays. The biological relevance of its suppressive activity could possibly be found in situations in which the concentration of α_1m is elevated, locally or generally. Patients with renal failure often have plasma levels of α_1m corresponding to the α_1m concentration used in the experiments of the present report [9]. Thus, α_1m might cause part of the impaired immune response commonly reported to be associated with uraemia (cf. Ref. 23).

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REFERENCES

- 1 Åkerström, B. & Berggård, I. Guinea pig α_1 -microglobulin. Isolation and properties in comparison with human α_1 -microglobulin. *Europ. J. Biochem.* **101**, 215, 1979.
- 2 Åkerström, B., Nilsson, K., Berggård, B. & Berggård, I. In search of α_1 -microglobulin on the lymphocyte surface. *J. Immunol.* **122**, 2516, 1979.
- 3 Berggård, I. Studies of the plasma proteins in normal human urine. *Clin. chim. Acta*, **6**, 413, 1961.
- 4 Bergstrand, H., Källén, B. & Nilsson, O. Effect of basic encephalitogenic protein and some peptides derived from it on the migration in agarose gel of leukocytes from patients with multiple sclerosis, other neurological diseases, or carcinoma. *Acta neurol. scand.* **50**, 227, 1974.
- 5 Chiu, K.M., Mortensen, R.F., Osman, A.P. & Gewurz, H. Interactions of α_1 -acid glycoprotein with the immune system. I. Purification and effects upon lymphocyte responsiveness. *Immunology*, **32**, 997, 1977.
- 6 Clausen, J.E. Tuberculin induced migration inhibition of human peripheral leucocytes in agarose medium. *Acta allerg.* **26**, 56, 1971.

- 7 Cooperband, S.R., Badger, A.M., Davis, R.C., Schmid, K. & Mannick, J.A. The effect of immunoregulatory α -globulin (IRA) upon lymphocytes in vitro. *J. Immunol.* **109**, 154, 1972.
- 8 Curtiss, L.K. & Edington, T.S. Regulatory serum lipoproteins: regulation of lymphocyte stimulation by a species of low density lipoproteins. *J. Immunol.* **116**, 1452, 1976.
- 9 Ekström, B. & Berggård, I. Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J. biol. Chem.* **252**, 8048, 1977.
- 10 Ekström, B., Peterson, P.A. & Berggård, I. A urinary and plasma α_1 -glycoprotein of low molecular weight: isolation and some properties. *Biochem. biophys. Res. Commun.* **65**, 1427, 1975.
- 11 Gahmberg, C.G. & Andersson, L.C. Leukocyte surface origin of human α_1 -acid-glycoprotein (orosomucoid). *J. exp. Med.* **148**, 507, 1978.
- 12 Greenwood, F.C., Hunter, W.M. & Glover, J.S. The preparation of ^{131}I -labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114, 1963.
- 13 Hedberg, H., Källén, B., Löw, B. & Nilsson, O. Impaired mixed leucocyte reaction in some different diseases, notably multiple sclerosis and various arthritides. *Clin. exp. Immunol.* **9**, 201, 1971.
- 14 Mortensen, R.F., Osmand, A.P. & Gewurz, H. Effects of C-reactive protein on the lymphoid system. I. Binding to thymus-dependent lymphocytes and alteration of their functions. *J. exp. Med.* **141**, 821, 1975.
- 15 Mortensen, R.F., Osmand, A.P., Lint, T.F. & Gewurz, H. Interaction of C-reactive protein with lymphocytes and monocytes: complement-dependent adherence and phagocytosis. *J. Immunol.* **117**, 774, 1976.
- 16 Ouchterlony, Ö. Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, **5**, 1, 1958.
- 17 Rocklin, R.E. Products of activated lymphocytes: leukocyte inhibitory factor (LIF) distinct from migration inhibitory factor. *J. Immunol.* **112**, 1461, 1974.
- 18 Seon, B.K. & Pressman, D. Unique human glycoprotein, α_1 -microglycoprotein: isolation from urine of a cancer patient and its characterization. *Biochemistry*, **17**, 2815, 1978.
- 19 Svensson, L. & Ravnskov, U. α_1 -microglobulin, a new low molecular weight plasma protein. *Clin. chim. Acta*, **73**, 415, 1976.
- 20 Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T. & Shikata, T. Tissue distribution of human α_1 -microglobulin. *J. clin. Invest.* **63**, 318, 1979.
- 21 Tejler, L. & Grubb, A.O. A complex-forming glycoprotein heterogenous in charge and present in human plasma, urine, and cerebrospinal fluid. *Biochim. biophys. Acta*, **439**, 82, 1976.
- 22 Tomasi, T.B., Jr. Serum factors which suppress the immune response. In Lucas, D.O. (ed.) *Regulatory Mechanisms in Lymphocyte Activation*. Academic Press, New York, 1977.
- 23 Touraine, J.L., Touraine, F., Revillard, J.P., Brochier, J. & Traeger, J. T-lymphocytes and serum inhibitors of cell-mediated immunity in renal insufficiency. *Nephron*, **14**, 195, 1975.
- 24 Weisbart, R.H., Bluestone, R., Goldberg, L.S. & Pearson, C.M. Migration enhancement factor: a new lymphokine. *Proc. nat. Acad. Sci. (USA)*, **71**, 875, 1974.

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